ANNUAL REVIEW OF MICROBIOLOGY

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VOLUME I

1947

ANNUAL REVIEWS, INC.
STANFORD, CALIFORNIA

61600

ANNUAL REVIEWS, INC.
STANFORD, CALIFORNIA

Foreign Agencies

London:

H. K. Lewis & Company, Limited
136 Gower Street, London, W. C. 1

Moscow:

Mezhdunarodnaya Kniga
Kuznetsky Most, 18

The Hague:

Martinus Nijhoff
9, Lange Voorhout

PRINTED AND BOUND IN THE UNITED STATES OF AMERICA BY
GEORGE BANTA PUBLISHING COMPANY

PREFACE

With the rapidly expanding interest in the study of microorganisms there has been an increase in the literature of microbiology to such an extent that no one can hope to keep abreast of the advances in the various fields. This is unfortunate because contributions to some specific aspect, applicable to other problems, may go unnoticed for some time. The recognition of similar situations confronting the research worker and the teacher in the fields of biochemistry and physiology led to the publication of the *Annual Review of Biochemistry* and the *Annual Review of Physiology*. Since these publications have proved to be of great value in their respective fields, a canvass of representative microbiologists was made which indicated that an *Annual Review of Microbiology* might be of similar worth.

The present plans envisage annual reviews of the more active fields of research, leaving the more quiescent areas to be summarized as developments demand. It is our hope that, by providing frequent résumés of current work in many fields, this new *Review* will answer the needs of microbiologists with diverse interests and will supplement the invaluable, more exhaustive treatments of the more limited topics appearing in journals such as *Bacteriological Reviews*. It is intended that the reviews will be critical appraisals rather than mere compilations of the literature. This implies that an individual reviewer may neglect some worthwhile contributions as not pertinent to his development of a topic. By soliciting the services of different reviewers for the same subject in succeeding years and with consequent variations in approach, the undesirable aspects of such treatment should be largely offset.

Suggestions for improvements will at all times be welcomed. Reprints of articles on microbiological subjects will be gratefully received and forwarded to the individual reviewers.

To those who have aided in this undertaking by their encouragement and criticism, and in particular to the authors of these reviews we extend our thanks. Their willingness to contribute without remuneration makes it possible to offer the *Annual Review of Microbiology* at the present low price. To Dr. D. D. Woods

and the *Annual Review of Biochemistry* we are indebted for permission to reprint the review on Bacterial Metabolism from Volume XVI of that publication. To the office staff of Annual Reviews Inc., we express our thanks for valuable aid throughout the development of this publication.

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MORPHOLOGY AND CYTOLOGY OF PROTOZOA

By D. H. Wenrich

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The literature on cytology of the Protozoa up to 1925 was considered by Bělař (1). The book by Calkins & Summers (2) which appeared in 1941, contains several chapters devoted to various aspects of morphology and cytology, hence the literature since 1939 to 40 is here given primary consideration. Due to the large area included and the severe limitations of space, a complete coverage has not been attempted. The material is arranged by major groups of Protozoa and the group names above the Family have been taken from Kudo's (3) *Protozoology*.

SARCODINA

Morphology

General features.—According to Mast (4) a monopodal *Amoeba proteus* typically consists of a zone of plasmagel surrounding a fluid plasmasol which moves forward, building up the plasmagel in front and being added to at the rear by transformation of plasmagel into plasmasol. Granular and other inclusions occur in both plasmagel and plasmasol. Outside all this is a thin hyaline fluid layer and a plasmalemma, or cell membrane. Many other amoebae show a similar organization.

Inclusions.—A critical examination of the granular and other inclusions of *Amoeba proteus* was made by Mast & Doyle (5, 6). Their list includes: (*a*) alpha granules, about 0.25 microns in diameter, about which little is known; (*b*) beta granules, about 1.0 micron in diameter, round or irregular in form, with the characteristics of mitochondria; (*c*) cytoplasmic refractive bodies which consist of an outer, neutral-red staining, osmiophile layer surrounding a central globular, carbohydrate shell filled with a fluid; (*d*) in the food vacuoles, vacuole refractive bodies which are homogeneous, osmiophile, neutral-red staining, derived from the food, and which disintegrate in the vacuoles; (*e*) fat globules which, within food vacuoles, consist largely of fatty acids, and when free in the cytoplasm consist of neutral fat; (*f*) crystals which originate

in food vacuoles and become isolated into small vacuoles by repeated divisions of the food vacuoles, and which eventually disappear leaving clear vacuoles in the cytoplasm; (*g*) on about 5 per cent of the crystals are blebs which have the properties of refractive bodies; and (*h*) various bacteria, molds, etc. The number and character of many of these inclusions vary greatly depending upon the kind of food eaten (7).

The cytoplasmic inclusions of the large multinucleate *Pelomyxa carolinensis* (*Chaos chaos*) are similar to those of *A. proteus*. Andresen (8) believed that crystals and refractive bodies fuse with food vacuoles but Wilber (9) provided additional evidence that they are formed in the food vacuoles. His cytochemical tests (10) indicated that the cortical material of the cytoplasmic refractive bodies is volutin or metachromatin. Hence they are probably not Golgi bodies, and Gatenby (11) reported absence of Golgi material in *Amoeba proteus*, *Nebela collaris* and *Arcella* sp.

The functions of the beta granules (mitochondria) seem uncertain. Neither Andresen (8) nor Wilber (9, 12) found them associated with food vacuoles and the latter doubted an excretory function, as did also Mast (13) after further study of *Amoeba proteus*.

In living *Entamoeba histolytica*, "digestive spherules" form near the nucleus, migrate to and attach themselves to ingested starch grains where they flatten out and coalesce with adjacent spherules. Erosion of the food body follows (14). The siderophile bodies attached to ingested starch grains in fixed and stained *Entamoeba muris* (15) are probably the same spherules. Chromatoid bodies of *E. histolytica* arise by coalescence of clear vacuoles which gradually become more viscous and plastic (14). In the marine amoeba, *Flabellula mira*, the entire vacuole system of the active and feeding animals is transient, smaller vacuoles coalescing with each other and with food vacuoles, producing larger vacuoles which eventually are defecated (16). It is probable that different kinds of amoeba have diverse vacuolar systems to serve their particular needs.

Reproduction

Agamy.—In amoeboid forms special interest attaches to the structure and division of the nucleus. A distinction may be made between the "trophochromatin" which stains intensely with iron haematoxylin, but takes no part in the formation of the chromo-

somes, and the "idiochromatin" out of which the chromosomes are formed. Often the latter is so dispersed that it stains hardly at all with iron haematoxylin and may even be Feulgen negative in the dispersed condition.

In the *Vahlkampfia* and *Hartmanella* types of small amoebae most of the trophochromatin is in a central endosome (karyosome, nucleolus). In the former type this divides during division and in the latter it disappears entirely. In *Amoeba terricola* the trophochromatin is in peripheral plaques and the idiochromatin is centrally located. Many other variations occur among these smaller amoebae (1).

In *Amoeba proteus* (*Chaos diffluens*) and *Pelomyxa carolinensis* (*Chaos chaos*), nuclear structure and nuclear division are much alike. Under the rather thick membrane of the discoidal nuclei is a layer of relatively large refractile granules which may be three to six hundred in number (17), and which are siderophile but Feulgen negative (18). In the large, ill-defined central area are fine Feulgen-positive granules and filaments which give rise to the chromosomes (18). In the early prophase the nucleus swells to about six times normal volume (17). As the central area condenses the peripheral granules are said to disappear (17) or to migrate to the central region and surround the condensed area where they give rise to the spindle fibers (18). Kudo (18) suggested one hundred and Short (19) about three hundred as the number of chromosomes for *P. carolinensis*, while Liesche (20) estimated five to six hundred for *A. proteus*. The nuclear membrane disappears at about metaphase and is reconstituted in telophase (17, 18, 20).

Amoeba lescherae (21) and *A. discoides* (22) resemble *A. proteus* in nuclear structure and division. In *A. dubia* (23) the spherical interphase nucleus contains siderophile but faintly Feulgen-positive granules which are evenly distributed through the achromatic matrix. During prophase they disintegrate and some larger achromatic bodies appear. As the nucleus assumes a barrel shape for the metaphase and anaphase, very fine chromosomes appear and divide on the spindle while the achromatic bodies collect at the poles. During telophase the achromatic bodies gradually disappear, and the chromatic granules reappear. The nuclear membrane persists (23).

The described formation of "chromidia" from the vegetative nucleus and the development therefrom of small encysted indi-

viduals which give rise to small amoebae does not seem to be fully demonstrated by the accounts (21, 22). Such small amoebae are quite different species (24) and the formation of nuclei out of "chromidia" may be doubted on theoretical grounds until proven beyond question.

There is much variation also in the nuclear structure and division of parasitic amoebae. In the genus *Entamoeba* the endosome is small; a layer of hypothecal granules against the nuclear membrane remains in place during division, and a relatively small amount of periendosomal chromatin contributes to the formation of the chromosomes. In *E. gingivalis* five chromosomes divide with prominant interzonal fibers between the daughters (25, 26). In *E. muris*, eight chromosomes divide and, as the daughter groups separate, about eight chromosome-like but Feulgen-negative interzonal bodies appear and stretch out on the elongating spindle (27).

In *Endamoeba blattae*, Meglitsch (28) found all parts of the nucleus to be Feulgen-negative except the chromosomes during their formation and division. These arise from the central nuclear area which is surrounded by a group of fairly large endosomes, and an outer zone containing small basophilic granules and somewhat larger refractive bodies. During metaphase and early anaphase the chromosomes are obscured by the great amount of material from the surrounding layers. The anaphase groups of chromosomes emerge from this mass and migrate to the poles while the nuclear membrane constricts in the middle. During the two or three precystic and several postcystic nuclear divisions there is a gradual reduction in the amount of trophochromatin. Species of *Endamoeba* from termites (29) show vegetative nuclei which resemble those of *E. blattae* during various of its cystic stages.

During nuclear division in *Endolimax nana*, a relatively large endosome disappears and about ten small round chromosomes divide on an intranuclear spindle which includes centrioles and a desmose. The nuclear membrane persists (30).

In the testate *Pontigulasia vas*, a hypothecal layer of endosomal granules or plaques disappears during the anaphase and reappears during telophase. The chromosomes arise from scattered granules and fibrils of the central area, forming a definite metaphase plate which divides in the usual manner with the nuclear membrane persisting (31). Other Testacea show a great variety of nuclear conditions (1).

Agamy in the Heliozoa is commonly by binary fission involving mitotic division of the nucleus with or without extranuclear centrosomes. Budding has been described (1). In the Radiolaria some species have large nuclei which divide with the formation of more than a thousand chromosomes. Multiple fission also has been described (1).

Syngamy.—Satisfactory evidence for syngamy in the Amoebina and Testacea is largely lacking. In the Foraminifera alternate generations are produced by multiple fission and by syngamy. Myers (32, 33) showed that for *Patellina corrugata* and *Spirillina vivipara*: (*a*) all nuclear divisions are mitotic; (*b*) meiosis is pregametic; (*c*) the haploid gametes are amoeboid; and (*d*) syngamy takes place within cysts in which two or more adult gamonts become associated. In *Discorbis patelliformis* and several related species he (34) found the gametes to be triflagellated, but in *Tretomphalus* (35) they were biflagellated as they are in *Polystomella* (36).

In the Heliozoa syngamy has been fully described for *Actinophrys sol* in which meiosis involves synapsis, tetrad formation, and two maturation divisions (1). Since syngamy seems to be so fully established for the Foraminifera and Heliozoa, its relative scarcity or absence in the other groups of Sarcodina is remarkable. Perhaps further studies will bring to light more satisfactory evidence for syngamy in these other groups.

SPOROZOA

Morphology

General features.—Although the Sporozoa are all parasitic and tend to form resistant spores, there is much diversity among them in morphology and details of life histories. All are supposed to nourish themselves by osmosis. The ectosarc of gregarines includes myonemes believed to be responsible for the characteristic bending, twisting, gliding and peristaltic movements. The cytozoic Coccidia and Haemosporidia are more simple in protoplasmic structure. The trophic stages of the Cnidosporidia are commonly multinucleate plasmodia, the coelozoic forms being typically amoeboid with a differentiated ectosarc. Ingestion of foreign bodies, though rare, has been reported for a myxosporidian (37).

The spores of Gregarinida and Coccidia are relatively simple,

consisting of a spore membrane (sporocyst) containing one or more sporozoites. In the Haemosporidia the sporozoites are not protected by a sporocyst, hence true spores are not formed. In the Myxosporidia sporoblasts are carved out of the multinucleate parent, thus segregating germinal from somatic material. Typically each spore results from the cooperative activity of six cells, two giving rise to the valves of the sporocyst, two producing the polar capsules, each with a coiled polar filament, and two being sporoplasm cells each with a gamete nucleus. These last eventually fuse to form an amoeboid zygote. In the Actinomyxidia the spore structure may be still more complicated and there is ternary instead of binary symmetry. The segregation of sporoblasts and the specialization of cells therein, especially as seen in the Myxosporidia, suggests a step in the direction of a metazoan type of organization.

Inclusions.—Various authors have studied inclusions in Sporozoa. For example, Joyet-Lavergne (38) recognized chondriome, Golgi apparatus, paraglycogen, lipoids and fats, albuminoid reserves, etc., in various gregarines and coccidia. Employing the ultracentrifuge in combination with cytochemical tests, Daniels (39) showed that the gregarines which she studied contained paraglycogen, fat, Golgi material, mitochondria, and chromidia, segregated to some extent into different layers. The neutral-red staining granules, however, were not displaced, thus demonstrating the distinctness between them and the Golgi material.

Reproduction

Agamy.—In the Telosporidia agamy is typically by schizogony (multiple fission) except in the Eugregarinina, in which numerical increase is brought about by the production of large numbers of spores. In some Coccidia the number of merozoites derived from a single schizont may be large; Hammond *et al.* (40) estimated 120,000 for *Eimeria bovis*. Noble (41) concluded that the Myxosporidia do not show true schizogony but may reproduce by budding or plasmotomy. Growth accompanied by nuclear division was called nucleogony.

Syngamy.—This is either isogamous or anisogamous. Even in isogamous gregarines, there are various ways in which the gamonts are differentiated, thus indicating a degree of sexual dimorphism (38). In the Coccidia and Haemosporidia, pronounced sexual di-

morphism is the rule, the microgametes being flagellated. In Gregarinida and Coccidia, both gametic and zygotic meiosis have been described. Most of the more recent accounts report zygotic meiosis. Among the Cephalina, zygotic meiosis has been claimed for *Actinocephalus parvus* (42), *Gregarina blattarum* (43) and for *Stylocephalus longicollis* (44). Among the Acephalina, *Zygosoma globosum* (45) and *Apolocystis elongata* (46) are said to have zygotic meiosis, while in *Filipodium osakii* (47), pregametic meiosis is described, thus making this a diploid species. Zygotic meiosis has been described for the coccidians, *Ovivora thelassemae* (48), *Adelina cryptocerci* (49) and *A. deronis* (50). In the latter species there is evidence for sex determination during meiosis, as there is also in Eimeria, a single oocyst of which can produce an infection (51).

During the formation of microgametes of malaria parasites, four to eight cytoplasmic filaments are thrust out from the microgametocyte; two chromosomes enter each of these. The chromosomes may fail to enter the filaments, thus producing sterile gametes (52).

In the Myxosporidia, chromosome numbers are generally low, the typical number being four (41, 53). According to Noble (41), in general, the single meiotic nuclear division is the last one in sporogony. The gamete nuclei would then be sisters and their fusion would constitute autogamy.

In the Actinomyxidia, Jirovec (54) found in *Sphaeractinomyxon ilyodrili* that eight slightly larger macrogametes and eight smaller microgametes are produced in a "pansporocyst." The last division is meiotic, reducing the diploid number from four to two. Fusion of these gametes in pairs produces eight zygotes which become sporonts.

In the Microsporidia, Weiser (55, 56) found a diploid number of about twelve chromosomes in *Plistophora chironomi* with a meiotic division preceding sporogony.

MASTIGOPHORA

Morphology

General features.—Flagellar structure varies somewhat. Typically each flagellum originates from a basal granule as an axoneme which extends out surrounded by a sheath. The tip of the axoneme may extend beyond the sheath. Reynolds (57) noted vescicles at

the tip of some flagella and Kirby (58), using the Bodian technique, found a knob at the outer end of flagella of *Trypanosoma brucei*, and also of *Trichomonas hominis* (59). Hair-like branches ("flimmer," "mastigonemes") on one or both sides of flagella have been described (60). Electron micrographs reveal an internal structure of parallel filaments in the flagella of *Trichonympha* (61). In electron pictures of the flagella of *Euglena* and *Astasia*, Brown (60) saw two longitudinal filaments, surrounded by a sheath which appeared to be a fine strand tightly coiled around the axonemes. He also saw evidence for "flimmer" on some flagella.

In the Polymastigina the anteriorly placed blepharoplast is the center of organization. From it originate the flagella, the rhizoplasts, the axostyles, the costas under the undulating membranes of *Trichomonas*, the crestas under the trailing flagella of the *Devescovininae* and the parabasal bodies of the Polymastigina.

The axostyle varies in size, shape, structure and stainability. Commonly cylindrical, it may be relatively thick and hyaline or thinner and chromatic, or even much flattened, as in *Saccinobaculus* (62). In the Oxymonadinae it is not only much elaborated in the cell body, but long fibrils from it extend forward as a rostellum (63). *Rostronympha magna* has an extensible proboscis which may reach 180 microns in length but its relation to the axostyle was not determined (64).

In its simpler forms the parabasal body may be round, rod- or V-shaped, but in the Devescovininae it may become coiled around the axostyle or more or less branched (65 to 69). The stainability of this structure is decreased by acetic acid, increased by chromium or osmium compounds, and it impregnates strongly with silver in the Bodian technique. It is Feulgen negative and does not divide (longitudinally) during cell division. In the haemoflagellates and some bodonids there is a rather compact body associated with the basal granules which is sometimes called a parabasal body but which should be called a kinetoplast. It appears to differ fundamentally from the typical parabasal body because it stains readily after most fixing agents, is Feulgen positive and divides during cell division (58). True parabasals are extensively developed among the Hypermastigina.

Using the Bodian silver technique, Kirby (59) found a new structure, the pelta, in *Trichomonas* (*Pentatrichomonas*) *hominis*. This is a smooth, flattened body, lying at the right of the blepharo-

plast and having a posterior projection. A pelta has also been found in *Trichomonas limacis* (70). Doubtless use of this technique will reveal similar structures in other flagellates.

Inclusions.—Hollande (71) and other French writers regard the Golgi material as consisting of either dictyosomes or the parabasal bodies, and reject the osmiophile cortex of the contractile vacuole as Golgi substance. Hollande did not find parabasals in flagellates with dictyosomes but did find the parabasal together with the osmiophile cortex of the contractile vacuole, as in *Chilomonas.* In this form Gatenby & Smyth (72) reported the osmiophile cortex as Golgi; but neither they nor Hall (73) saw the parabasal body and the latter considered small scattered osmiophile globules to be the Golgi materials. In addition to the structures mentioned above, *Chilomonas* has extensive deposits of fat and starch and two ovoid birefringent bodies. Hollande (71) insists there is no transition between chondriome and Golgi material.

In representatives of the Phytomonadina, Hollande (71) described the chondriome as peripherally placed filaments which may or may not anastomose into a network. In *Cryptomonas* there were numerous small chondriocont... He rejected Hall's (74) identification of scattered cytoplasmic spherules as mitochondria. In colorless euglenoids, the chondriome varied from simple chondrioconts to a net or system of bands parallel to the cuticular striae. Much variation occurred even in the same species. He was unable to find chondriome in green euglenoids where Hall (74) reported scattered granular mitochondria. Differences in interpretation are doubtless based upon subjective as well as technical considerations.

"Trichocysts" have been described for various flagellates by Hollande (71) and many others. In *Chilomonas* they are more prominent on the walls of the "pharynx" but occur also just under the body surface. Hollande believes that they provide material for the cyst wall and also serve for saltatory movements. In the euglenoids somewhat similar subcuticular muciferous bodies have frequently been reported and apparently provide material for cysts (71). In *Gonyostomum semen* the trichocysts may discharge mucoid filaments to a distance of five hundred microns (75). In the dinoflagellate, *Polykrikos schwartzi*, not only trichocysts, but also very complicated "cnidocysts," each with a long coiled filament, in addition to parabasal bodies, mitochondria, Golgi material, vacuome, fat globules and glycogen have been described (76). In the green

flagellates, of course, the chromatophores, and various reserve food bodies are also found.

Reproduction

Agamy.—Nuclear division in flagellates is normally mitotic. Often the nuclei are of the vesicular type with a central endosome surrounded by the idiochromatin from which the chromosomes arise. In certain hypermastigotes and other flagellates the chromosomes are attached to the nuclear membrane by chromosomal fibrils, even in the interphase (62). In some flagellates the endosomes disappear during division but in euglenoids they persist and divide.

Chromosome numbers vary from two to several hundred (77). In Phytomonadina, recorded haploid numbers vary from eight to thirty-two. In the Euglinoidina from twelve to as many as two hundred have been reported and in the Dinoflagellata the number may be even higher. Simpler endozoic flagellates usually have low numbers but in the Hypermastigina there may be from two to sixty. Species of *Spirotrichosoma* are reported to have twelve, twenty-four, forty-eight and sixty chromosomes, representing polyploidy (78).

Division of the chromosomes is generally longitudinal, but in some cases, as in the Oxymonadinae (63) they are not sufficiently individualized to determine this point. There is evidence for division in the anaphase or telophase instead of in the prophase and in euglenoids both methods have been described (71).

In most cases the nuclear membrane persists during division. The origin of the division spindle may be intranuclear, as in the Oxymonadinae (63), or mostly extranuclear. It may apparently be absent, as in the euglenoids (71). In *Barbulanympha* the elaborate achromatic figure includes a central spindle between the asters. This pushes in on the anterior surface of the nucleus which becomes depressed by it. Some astral rays join the intranuclear chromosomal fibers at the nuclear membrane which persists throughout division. The fibrils from chromosomes to asters are thus of double origin, part from the chromosomes and part from the asters (62, 79).

When the cell divides, two sets of organelles must be provided for the two new daughters. As Kirby (58) points out, newly formed individuals may obtain their organelles by direct transfer from the parent, by division, or by neoformation. The evidence as to method

of acquisition is not consistent, even for the same type of organelle. Basal granules divide, but apparently flagella do not. Parental flagella may be dedifferentiated completely, as in *Lophomonas* (80), or may persist and be passed along to daughters as in the Trichomonadidae (*Gigantomonas herculea*, with its amoeboid reproductive phase is an exception (81)) and in most of the Hypermastigina. The costa of *Trichomonas* persists for one daughter but the parental cresta of the Devescovininae disappears entirely. The axostyle dedifferentiates and must be replaced by a new outgrowth from the blepharoplast. Division of the cell body is generally longitudinal.

Syngamy.—In the Phytomonadina syngamy is well known, meiosis being zygotic. Cell fusion has been described for certain euglenoids (82, 83) but nuclear details are not complete.

Evidence for syngamy in animal flagellates has been largely lacking, but in the Hypermastigotes and other flagellates of the wood-roach, *Crytocercus*, Cleveland (84) has found syngamy, including autogamy, occurring in connection with the molting of the host. Meiosis is zygotic. In these flagellates doubling of the chromosome number and meiosis may be correlated with the behavior of the centrosomes (78). Perhaps syngamy in other animal flagellates will be found.

CILIOPHORA

Morphology

General features.—In contrast to the traditional view that a cilium is an axoneme arising from a basal granule in the ectosarc and projecting from the body surface surrounded by a sheath of different material, the electron microscope reveals a series of constituent longitudinal fibrillae (61). With the addition of the shadow casting technique, about eleven fibrils were clearly seen to compose the cilium of *Paramecium* (85). There was no visible enclosing membrane.

Electron micrographs also show that the discharged trichocyst of *Paramecium* consists of a sharp-pointed opaque endpiece and a spindle-shaped shaft with cross striations having a periodicity of about 550 Å. There is evidence of a pattern four times larger (2200 Å). Shadow casting shows this shaft to be flat. There are indications of longitudinal striations (85, 86).

In most of the ciliates carefully studied, the basal granules of the cilia in each row are connected by a longitudinal fiber which,

as shown by Worley (87) for *Paramecium*, regulates metachronal activity. Cross connections between rows, especially at the poles, may provide for more complete coordination. Since the pioneer work of Sharp (88) on *Diplodinium*, many accounts, especially by American authors, have recorded a complete system of connecting fibrils with a coordinating center or "motorium" (89).

Silver inpregnation techniques have revealed extensive systems of pellicular and subpellicular fibers with reticulate or other patterns, making up the "Silberliniensystem," "argyrome," etc. In the contractile species myonemes and neuronemes have been described (89). There is no general agreement as to the functions of these fibrillar structures, and Villeneuve-Brachon (90), in a study of about thirty species of the Heterotricha, reaffirms Chatton's rule of the autonomy of the ciliary rows and does not subscribe to the neuromotorium concept.

Inclusions.—Apparently all the inclusions commonly recognized in Protozoa have been reported for ciliates. These include mitochondira, Golgi apparatus (dictyosomes, fatty acid bodies, intermediate lipoid bodies, secretory bodies, osmiophile cortex of the contractile vacuoles), neutral red granules (segregation granules, digestive granules), reserve bodies (protein, lipoid, carbohydrate), pigment granules, etc. (91).

Mitochondria are usually small bodies scattered through the cytoplasm or concentrated near the body surface and associated with vacuoles and other structures (90 to 93). Golgi apparatus includes the osmiophile cortex of contractile vacuoles as early emphasized by Nassonov (94) and supported by Gatenby (11) and Smyth (95, 96), as well as osmiophile granules and dictyosomes (90 to 93). The osmiophile cortex is absent in sea water ciliates, *Nyctotherus* and certain fresh water species (11, 95, 96). In some ciliates it divides with the vacuoles but does not in *Vorticella* (95). Scattered osmiophile bodies are present in some ciliates with and some without an osmiophile cortex surrounding the contractile vacuole (95). Smyth (95, 96) believes that osmiophile material (Weigl or Kolatchew techniques) whether or not associated with the contractile vacuole constitutes the Golgi material.

Reproduction

Agamy.—Longitudinal fission occurs in the Protociliata, transverse fission in the Holothricha and Spirotricha, but an apparent

longitudal fission is seen in the Peritricha; budding occurs in the Chonotricha and Suctoria. The micronuclei divide mitotically and the macronuclei amitotically. During division the macronucleus often undergoes a reorganization. In some ciliates, for example, the Colpodidae (97), macronuclear division is accompanied by a visible discharge of material into the cytoplasm.

Cytoplasmic fission may follow three patterns (98). (*a*) In *Paramecium*, production of new cilia and trichocysts is limited to the division zone in the middle of the parent, each daughter receiving relatively unchanged one-half the parent. (*b*) In *Condylostoma*, the large peristome undergoes partial dedifferentiation to be rebuilt for the anterior daughter, while the posterior daughter is provided with a new one. The two new bodies are shaped from the parent without the growth of new ends as in *Paramecium*. In *Oxytricha*, the parental cirri are all replaced by new ones, the latter first appearing as a rudimentary group in each half of the parent. One may raise the question as to whether the cilia and trichocysts of *Paramecium* which are passed along unchanged from parent to offspring are endowed with "immortality," since encystment, with dedifferentiation, is not characteristic of this genus. In some other members of the Holotricha, for example, *Cryptochilidium bermudense*, new cilia arise all over the body by division of the basal granules of the parent (99).

Although various formed elements of the cytoplasm may apparently be handed on unchanged from parent to offspring during fission, in general there is dedifferentiation on encystment and redifferentiation on excystment (100). Just what structures are retained? Chatton and his associates (101) have long maintained the principle of the "continuité génétique des cinétosomes" The. persisting basal granules, by division, provide all required new cilia and trichocysts (90). Even in the Hypotricha, where Klein (102) believed that new basal granules could be formed *de novo* from the silver line network, Chatton & Seguela (103) have attempted to trace basal granules for new cirri from those of the parent.

Syngamy.—In the Ciliophora, syngamy typically takes the form of conjugation, except in the Protociliata where complete fusion of unequal gametes has been described. Partial reviews of the subject are provided by Diller (104), Woodruff (105), Turner (106) and Finley (107).

Typically, conjugation involves the adherence of two individuals followed by meiotic micronuclear divisions, mutual exchange of migratory pronuclei, synkaryon formation, and after separation, absorption of the old macronucleus and the development of a new nuclear apparatus from the products of division derived from the synkaryon. There are many modifications of this process. In *Paramecium*, endomixis (108, 109) was described as nuclear reorganization without cell fusion, but Diller (110) showed that it included autogamy and hemixis, the latter a regeneration of new macronuclei out of fragments of the old. Sonneborn (111) believed that after some forty fragments of the macronucleus of *P. aurelia* had been produced, each of these could develop into a new macronucleus when segregated by successive cell divisions, suggesting extensive polyploidy of the macronucleus. Autogamy may occur in *P. caudatum* even when two animals are joined together (112), and when a third individual joins a pair of conjugants in *P. bursaria* (113, 114).

Many cytological variants have been found among conjugants and exconjugants (115), including hypoploidy and hyperploidy in *P. caudatum*, exchange of parts of macronuclei between conjugants in *P. trichium* (104), and reconjugation (116). In *P. bursaria*, Chen (117, 118, 119) noted extensive polypolidy, even within the same mating type. Amicronucleate races conjugated with micronucleate, each conjugant receiving one pronucleus or "hemikaryon." In the same species, Wichterman (120) observed failure of one migratory nucleus to pass to the other conjugant, so that one exconjugant was "haploid" and the other "triploid."

In most ciliates, conjugants are of the same size, although they may be smaller than normal. In the Peritricha, typical conjugation is between normal-sized macroconjugants and much smaller microconjugants. The latter are formed by successive divisions or by budding from ciliates of normal size. In the Vorticellidae the nuclei and endoplasm of the microconjugant may enter the macroconjugant before nuclear divisions take place, as in *V. microstoma* (121), or the miconjugant may enter completely as in *Opisthonecta* (122), or other variations may occur. In the unstalked Urceolariidae (123, 124) the difference in size between conjugants is less marked, but the behavior is comparable to that in the Vorticellidae. In all cases both conjugants undergo meiotic micronuclear divisions, but only one synkaryon is formed and this functions for

the macroconjugant, the microconjugant being discarded to a greater or less extent.

As noted by Finley (107), most authors record that "reduction" of chromosome numbers takes place in the second meiotic division. The number of division products derived from the synkaryon in different species may be two, four or eight, but occasionally more are formed, either regularly, as in *Kidderia*, where sixteen are produced, or as a variation from the normal. In a series of processes as complex as those involved in conjugation, one might expect to find a great deal of variation.

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ANTIGENIC VARIATION IN PROTOZOA AND BACTERIA

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The following article is presented in an attempt to give a summary to some recent work in immunology which may be of special interest to students of microbiology. The work deals with the phenomenon of antigenic variation which has been observed as a frequent occurrence in certain species of bacteria and protozoa and as an uncommon occurrence in other species. Rather than attempt a review of all the reports of antigenic variation in the literature of microbiology, the author has chosen to relate the studies made on a few selected groups of microorganisms in order to correlate from them certain considerations which may appear to be operative whenever antigenic variation occurs in individual cells or in large populations of cells. It is to be emphasized, therefore, that many additional facts of antigenic variation are to be found not only in the works which are reviewed in part, but also in numerous reports to which no reference is made.

One of the most striking things about antigenic variation in the bacteria and the protozoa is the difference in stability of variant forms of different species of organisms. A number of species are very stable while maintained under constant condition of culture. The pneumococci, for example, show no detectable change in the immunologically specific carbohydrate characteristic of the capsular element of the cell when maintained by rapid subpassage through mice or in *in vitro* culture in meat infusion media enriched with serum or serous fluids. Similarly, the rough colony variant of the pneumococcus, once secured and stabilized by growth in poorer media, shows no detectable variation in over-all antigenic character. On the other hand some species are found to be in some stage of antigenic change almost always during cultivation in media of various sorts. Many species of the genus *Salmonella*, for example, show antigenic variation in the components of the body of the organism and the components of the flagella irrespective of the media in which they are grown.

Different species of microorganisms also show a marked differ-

ence in the extent to which variation occurs. Some species vary with a few antigenic types while others show a very wide range of variations. For example, some twenty or more antigenic variants of a single original brood of trypanosomes have been observed to occur during the course of recoveries and relapses in animals infected with these parasites [cf. Taliaferro (1)] while *in vitro* experiments somewhat similar in character have shown only three or four variants present in strains of certain bacteria and other organisms.

In spite of certain technical and interpretative difficulties of the various tests applied in the studies reviewed here a great deal has been learned about the changes which take place antigenically during continued growth of microorganisms in one environment or another. An attempt will be made to draw together various facts learned in the study of different species, and to garner from them some consideration of the cellular mechanism which controls the over-all character. It is to be hoped that the liberties taken in comparing the data presented from studies on widely separated taxonomic groups will not seem wholly unwarranted.

Pneumococcus Variation

Among the most interesting of all recent developments in antigenic variation has been the studies in directed variation between immunologically distinct types among the pneumococci. These organisms ordinarily show very marked antigenic specificity associated with the polysaccharide of the capsule when first isolated from human beings or when maintained under special laboratory conditions such as subpassage through mice. On the basis of serologic reactions seventy-five varieties of these type specific forms are now recognized according to Eddy (2). The type specific forms gradually degenerate, or otherwise lose type specificity, when grown in ordinary meat infusion media devoid of serum or other fresh body fluids. This change is accompanied with a loss of capsule and a change in colony form from smooth to rough colonies. The sharply distinct antigenic pattern observed in the smooth colony of encapsulated organisms is not preserved in the rough colony of unencapsulated variants. If the rough derivatives from any particular smooth parent culture are different antigenically from the rough derivatives of smooth cultures of other types no report has been made of the fact. Nevertheless, the rough derivatives from differ-

ent specific types are apparently different in some respects for, according to the experience of many workers, each rough derivative culture regains its previous antigenic distinction when it reverts again to the smooth form. Reversibility from the rough form to the original type-specific form may be obtained from some R cultures grown in meat infusion media enriched with antiserum or normal serum or by rapid subpassage through mice.

Griffith (3) induced a change in a pneumococcus from Type II to Type III by planting in mice a small amount of R derivative from Type II together with a large amount of heat killed Type III culture. The organisms cultured from the animals so treated were uniformly of the Type III variety. Later Dawson & Sia (4) were able to induce a similar transformation *in vitro*. They incorporated a large amount of heat killed Type III organisms and antisera for the R form in the fluid media in which the rough derivatives were inoculated. Further evidence that the transformation was brought about by nonviable components of the heat killed cultures was secured by Alloway (5, 6) who succeeded in bringing about transformation with extracts from Type III cultures which had been rendered free from all cells and cellular debris by passage through Berkefeld filters. Still later, Avery, MacLeod & McCarty (7) crowned these remarkable achievements with the isolation of a highly pure, polymerized form of desoxyribonucleic acid[1] from a Type III culture which, when combined suitably with other reagents, induced the development of a Type III strain from the R variant of a Type II strain. These authors presented their results as a representative model of the possibilities of transformation between any of the specific forms of pneumococci. The isolation of transforming agencies from Types II and VI pneumococci was reported by McCarty & Avery (8). The possibility of unlimited transformation between any of the seventy-five types of pneumococci recognized by Eddy (2) seems implicit in the work reviewed here. However, it should be noted that no evidence has been produced to show that similar transformations occur in nature.

[1] The authors reported that the data obtained by several methods of analysis, including electrophoresis, ultracentrifugation and ultraviolet light spectroscopy as well as various chemical, enzymatic and serologic tests, indicate that the substance contains no demonstrable protein, unbound lipid or immunologically active polysaccharide. However, Mirsky, according to Muller (44), has given reasons for inferring that the agent may have protein structures bound to the nucleic acid.

The nature of the R forms of pneumococci which are reconvertible to an S form of type heterologous or homologous to the original S form of the R derivative is a matter of considerable interest. Dawson (9) noted that R derivatives from either Type II or Type III parents could be converted to other types and that a single R variant could be converted serially from its original type-specific form to each of three other type-specific forms provided it was degraded to the R form between each conversion. Spontaneous reversion to original type in culture medium devoid of transforming agencies occurs according to Alloway (5, 6) if the medium contains suitable amounts of serum from normal sheep, rabbits, guinea pigs, horses or human beings, or pleural or ascitic fluid from the human. Alloway (5) stated that these body fluids differed markedly in their anti-R titre and that the observations made seemed to indicate that some property other than antibody in the anti-R serum was responsible for the reversion of R form to S forms. Alloway (6) further noted that some R variants were much more resistant to transformation than others, and Avery, MacLeod & McCarty (7) reported that among four different types of colonial variants isolated from a single R form only one was susceptible to action of the transforming agency. The fact that some R variants retain the capacity for reversion to original type in the absence of transforming stimuli would indicate that some of the agency concerned with regulation of type specificity lingers in those cells. However, it must be very little for Dawson (10) noted that very large amounts of heat killed R culture, capable itself in viable form of reversion, failed to induce transformation of small quantitites of living R cells when both were inoculated simultaneously.

Data scattered through the various reports cited above seem to indicate that the transforming agency, though not itself the antigen responsible for type specificity in smooth cultures of pneumococci, is, nevertheless, most concentrated in the highly type-specific forms and least concentrated in the nonspecific rough forms. These data also suggest that during the course of change from heavily encapsulated to unencapsulated forms there is a loss of transforming substance more or less paralleling the loss of capsular material. McCarty & Avery (8) point out that the individual desoxyribonucleates isolated from different types of pneumococci are probably different from one another in some respects of chemical structure although they have been unable to show any differ-

ence between them other than the types of transformation they induce. Additionally, McCarty (11) in a discussion of this work stated that a desoxyribonucleic acid could be extracted from an R variant of pneumococci which is entirely similar to the derivative of Type III pneumococci except that it is wholly inactive in producing transformations.

It should be pointed out that the experimental procedures involved in transforming one specific type of pneumococcus to another has been unsuccessfully applied to other organisms. Thus, Cherry, Barnes & Edwards (12) were unable to induce transformation in four monophasic strains of *Salmonella* by growth in media containing antiserum suppressive for the flagellar antigen and heavy suspensions of heat killed cultures which had flagellar antigens of a different character from those of the monophasic culture grown.

Again it should be stressed that a careful examination of various reports on transformation of pneumococcus type does not reveal a single instance in which the R form was converted to any type other than its original type in the absence of transforming stimuli. Furthermore no reports have been made of it if R variants, fully devoid of capsular antigen, ever revert to original type in the presence of sufficient transforming agency of other type to induce change in some of the cells, although Dawson (9) reported that some cells in variant cultures which were intermediate between the S and R forms reverted to original form in the presence of vaccines of heterologous types and that S forms could not be directly transformed to other types of S form. It therefore appears that pure R variant cells only are sensitive to transformation to another type and that they have little of the agency which controlled their original type.

Flagellar Variation in *Salmonella*

Several types of antigenic variation have been described for various species in the genus *Salmonella*. These organisms show changes in the body of the organism (somatic, or O = Ohne Hauch, variation) on the one hand, and, on the other hand, in the flagella of the organism (flagellar, or H = Hauch, variation). The flagellar variations are ordinarily independent of somatic variations and will be so described.

A very large portion of *Salmonella* species show a frequent and

consistent variation in the group of antigens which characterize their flagella. Under ordinary cultural conditions the variant forms found are limited usually to two general types. Thus, for example, a plate culture of *Salmonella cholera suis* may be composed of colonies of both antigenic varieties. At an earlier time all colonies and cultures predominantly of one sort were referred to as being in the specific phase, while colonies and cultures of the other sort were referred to as being in the group or nonspecific phase. Recently the more suitable terms, phase 1 and 2, have come into general usage. All cultures which show the coexistence of phase 1 and phase 2 varieties during cultivation in ordinary media are called diphasic strains or species according to Andrewes (13). In distinction to the more commonly occurring diphasic species some strains and species of *Salmonella* show little or no variation in flagellar antigens during growth in ordinary culture media. These are referred to as monophasic.

No difficulty is encountered in demonstrating phase variation in the usual diphasic strains of *Salmonella.* Colonies of phase 1 and of phase 2 character are commonly found among the petri dish isolates from a young colony irrespective of the phase character of the parent colony. One may also demonstrate the presence of phase 1 and phase 2 individuals in most populations developed quickly from a single parent cell. In some instances one phase or the other may be predominantly present in a diphasic culture. The demonstration of the less prevalent phase in these cases is most readily accomplished by the addition of an antiserum which suppresses the dominant phase and allows the development of the less prevalent phase.

Whether or not any of the *Salmonella* strains ordinarily contain more than two phase variations of flagellar antigens is a question of considerable interest. There can be no question, however, that more than two may be developed when certain strains are cultured suitably in antiserum. Thus by applying the technique of Wassén (14, 15) Bruner & Edwards (16) demonstrated that *Salmonella paratyphi A* grown in media containing suppressive antisera of one sort or another could show as many as four different phases. The interrelationship between the four variant forms and the reversibility from one form to another is illustrated in Fig. 1.

The monophasic strains of *Salmonella*, in distinction to the diphasic strains, ordinarily show a single antigenic form of the

flagella among all colony isolates. However, it is clear that these forms either contain suppressed variant forms normally or may be induced to develop variant forms by suitable experimentation. The isolation of additional phase variants from numerous phase 1 monophasic strains and phase 2 monophasic strains have been reported by Kauffmann (17), Kauffmann & Tesdal (18), Edwards (19), Edwards & Bruner (20, 21, 22) and Bruner & Edwards (23). While the variant strains isolated by Kauffmann (17) from *Eberthella typhi* appeared to be irreversible monophasic variants a similar variant isolated by Edwards & Bruner (20) reverted to

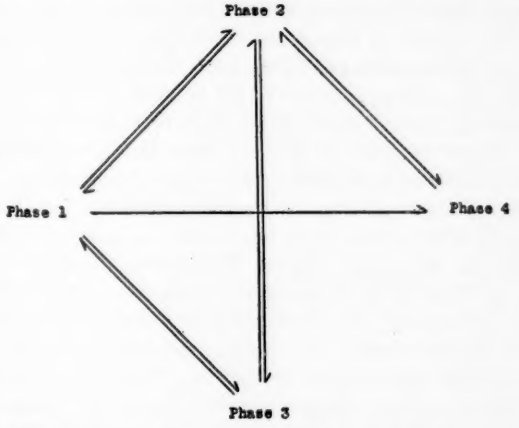

FIG. 1.—REVERSIBILITY OF PHASES OF *S. paratyphi A* 228. ARROWS INDICATE DIRECTION IN WHICH VARIATION OCCURRED [Bruner & Edwards (16)].

original form when cultivated in medium containing antiserum for the variant form.

It is not at all surprising that the liability to variation shown by the *Salmonellas* has aroused speculation on its possible importance in the phylogenetic development of species. Complex combinations of flagellar antigens, which for the genus numbers more than sixty different components, accounts for the distinction of a very large number of species, each with its own peculiar antigenic composition. It has been suggested that many of these various species may have developed from some common ancestral form by

infrequent variation which involved the loss of one or more antigenic component [cf. White (24)]. A very recent demonstration of this possibility was reported by Edwards (25) in which a culture *S. hormaechi*, demonstrated by the usual method to be pure, continued for quite a while during ordinary culture to show daughter colonies only of z_{30} and $z_{30}z_{31}$ types, then after a lapse of one year began to show only colonies of the $z_{30}z_{31}$ and z_{31} types. It is interesting to note here that during the early period of study the original culture on serial plating continued to produce colonies about 5 per cent of which contained only z_{30} and 95 per cent contained $z_{30}z_{31}$, while during the later period of study z_{30} colonies were no longer produced, and instead about 12 per cent of the colonies were z_{31}. This report is important for the fact pointed out by its author, that it illustrates that a complex culture can dissociate two loss variants, z_{30} and z_{31}, both of which are stable, and that the loss variants are similar to cultures found in nature. An equally striking instance of loss variation is that reported by Edwards & Bruner (26). These authors described a new type of *Salmonella*, *S. salinatis*, then by cultivation in medium containing selected antisera saw it produce a variant form which was indistinguishable from the well-known San Diego type of *Salmonella*. Edwards & Moran (27) reported success in changing all of nine cultures of *S. simsbury* to forms serologically indistinguishable from *S. senftenberg*. It appears then that rather remarkable variations ranging between forms previously described as species may be found to occur with suitable laboratory technique. It seems doubtful, however, that any single primordial organism may have been sufficiently complex to have developed through occasional loss variations alone into the large number of rather stable forms now recognized. However, one is hard pressed to find experimental evidence which directly supports any other concept with regard to this group of bacteria. On the other hand the demonstrations by Edwards & Moran (27) that the variation, b→enx, in *S. minnesota* occurs only through an intermediary antigen z_{33} (b→z_{33}→enx) and by Bruner & Edwards (16) that reversion from the artificial phase 4 of *S. paratyphi A* to the natural phase 1 occurs only through the intermediary phase 2 (phase 4→phase 2→phase 1) may be taken as a suggestion that a rather extensive possibility for variation through numerous stable intermediate forms is possible by means other than loss variation alone in the *Salmonellas*.

Trypanosome Variation

Particularly striking is the very extensive range of antigenic variation observed in pure lines of pathogenic trypanosomes propagated in mice, rats and guinea pigs by Ehrlich, Levaditi, Schilling, Franke, Ritz, and others [for an excellent, comprehensive review of this work see Taliaferro (1)]. An initial infection is usually fatal to mice unless they are treated with trypanocidal drugs but in the guinea pig a series of recoveries and relapses intervene between the initial infection and death. However, if mice are treated at appropriate times with adequate doses of arsenicals, antimonials, or other trypanocidal drugs they show a sequence of infections and recoveries which is similar to that observed in guinea pigs without drug treatment. In both cases the broods of parasites found to dominate most of the relapses are antigenically distinct from each other and from the brood of the initial infection. In both cases recovery from the initial infection and each succeeding relapse is attended with the appearance of trypanolytic antibody specific for the parasite brood of the preceding paroxysm. In an extensive series of experiments in which trypanosome broods found in each succeeding relapse in treated mice were maintained by rapid subpassage through untreated mice Ritz, according to Taliaferro (1) was able to demonstrate the appearance of seventeen immunologically different variants in a single mouse and in over six hundred mice found twenty-two antigenic variants in all to have developed from an initial infecting strain. Ritz (28) later showed that an apparently inexhaustible succession of immunologically distinct variants may appear in a single animal. This work, incidentally, is of especial interest since the strain of *T. brucei* used was a "pure line" derived from a single trypanosome.

Several of the earlier workers, including, according to Lourie & O'Connor (29), Ehrlich, Ritz and Schilling and co-workers, noted that relapse broods of trypanosomes were sometimes compound strains in the sense that each individual had the antigens characteristic of two or more immunologically distinct strains, or in the sense that the broods were heterogeneous mixtures of several antigenic varieties. Levaditi & Muttermilch (30) for example, found in microscopic examination of several *in vitro* parasite-antiserum mixtures that some individual parasites were insensitive to the antiserum while the remainder were rapidly lysed. Lourie &

O'Connor (29) more recently have reported several instances in which the relapse strains of *T. rhodesiense* studied were composed of individuals of two distinct antigenic types. Strain 11, for example, was found in *in vitro* experiments to be composed of about equal numbers of individuals of type "b" and individuals of type "c." While each of ten single cell isolates from the parent stem strain developed into immunologically identical and antigenically homogeneous broods when inoculated into different mice, one single cell isolate from the relapse strain 11 developed into a homogeneous brood of "b" type and another developed into a homogeneous brood of "c" type. In another instance these workers found a relapse strain to be composed of individuals of at least three distinct antigenic types. It is interesting to note here that the ratio between the numbers of each type found was variable in different mixed strains. One mixed strain, for example, was found to be composed of individuals of types "b" and "c" in about equal numbers, while in another strain the proportion of the two types was found to be about 10-1. From this and other data, Lourie & O'Connor (29) suggested that a large number of antigenic types might appear and persist during the development of a brood from a single parent stem and that the ratio between the most prevalent and least prevalent types in the brood might be as high as 500-1. It appears further from the work of these authors that the occurrence of antigenically heterogeneous broods of trypanosomes is not particularly uncommon for they found seven of their twenty-two relapse strains to be so mixed.

Borrelia Variation

The association of the relapse phenomenon and antigenic variation in trypanosomes is also apparent in natural and experimental infections with the *Borrelias*. Cunningham (31) found that the organism, *Borrelia carteri*, occurred in two antigenic varieties in human infections of relapsing fever in India. He was able to show through experiments in squirrels and monkeys that these two strains were antigenic variants of each other. As a rule strain B appeared in the first relapse in animals following an initial attack with strain A and strain A, as a rule, appeared in the first relapse in animals following an initial attack with strain B. This worker also noted the development of three additional antigenic varieties of *Borrelia carteri* during the course of prolonged intermittent

infections in squirrels. These additional strains were considered to be antigenic subgroup variants of the strains A and B.

Meleney (32) presented clear evidence of the development of six immunologically distinct strains of the organism, *Borrelia recurrentis*, when a single human strain was passed through a series of splenectomized squirrels in which the relapsing type of infection developed. This worker found some consistency in the sequence with which one strain followed another in succeeding relapses. In all of the experiments strain II was the only one to appear in the first relapse from an original infection with strain I and strain III was the only one to appear in the second relapse. However, when the first infection was produced with strains other than strain I the sequence was less consistent. This worker (32) noted further that while strains I and II were quite distinct from each other they reacted with some of the antisera to other strains. Thus strain I reacted with some of the antisera to strain III but not with any of the antisera to strain II, while strain II reacted with some of the antisera to strain IV but not with any of the antisera to strains I and III. This result, as will be seen later, may have been due to the appearance, simultaneously, of two or more antigenic types in the brood of infecting parasites.

Through a very extensive study of *Borrelia carteri*, Cunningham, Theodore & Fraser (33) were able to identify seven antigenic variants in addition to the two antigenic phases described earlier for the organism by Cunningham (31). This work demonstrated beyond question that the organism had the potentiality of at least nine different immunologic types, each a variant form of the other. The diagrammatic summary of the phase relationship of eight of these strains presented by Schuhardt (34) may be seen in Fig. 2. Cunningham, Theodore & Fraser (33) further reported that there was no pathogenic or morphologic difference between the several antigenic variants isolated, save two. The eighth and ninth strains, "H" and "I," were somewhat different from the other strains in that, in all cases, a marked delay occurred between the time of inoculation with the passage strains in the host and the appearance of symptoms of infection. All of the strains were, however, sharply distinct immunologically for, with only one exception, no cross reactions were noted between any of the strains as long as they remained pure. The exceptional case was one in which a recovered animal showed antibodies for several of the antigenic types when

its serum was tested long after the initial attack in spite of the fact that no evidence of intercurrent relapse was seen.

The difference in degree of specificity among the variant strains of *Borrelia recurrentis* reported by Meleney (32) and among the

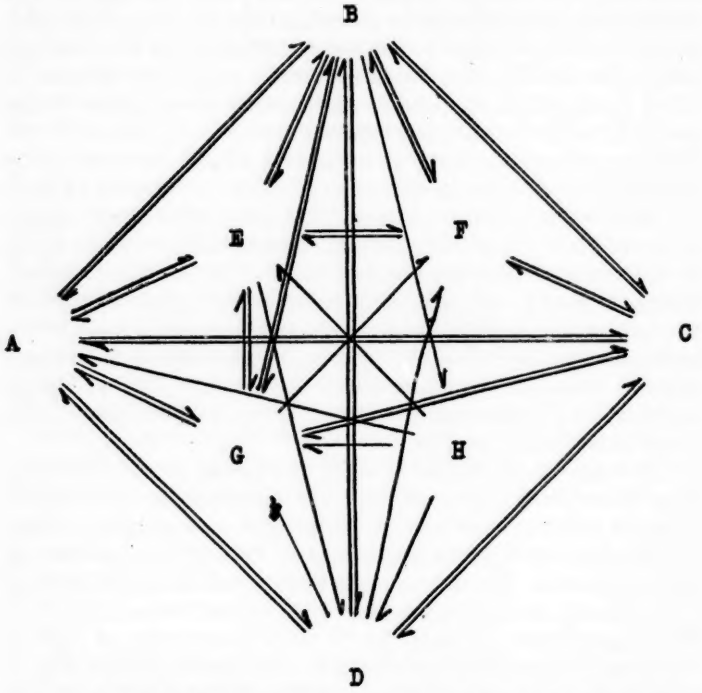

FIG. 2.—DIAGRAMMATIC SUMMARY OF DEMONSTRATED ANTIGENIC PHASE RELATIONSHIPS OF THE SPIROCHETES STUDIED BY CUNNINGHAM, THEODORE & FRASER (33). ARROWS INDICATE DIRECTION OF INDUCED (RELAPSE PASSAGE) OR NATURAL (ONSET PASSAGE) PHASE VARIATIONS [SCHUHARDT (34)].

strains of *Borrelia carteri* reported by Cunningham, Theodore & Fraser (33) is not explainable from the information given in their papers. It should be pointed out, however, that these are different species of organisms and for that reason may not be expected to act alike in all respects of variation capacity. It should be noted also that the work of the former author was necessarily done with

splenectomized squirrels while the work of the latter authors was done with normal squirrels. However, in spite of a failure to see cross reactions between the antigenic variants of *Borrelia carteri*, the latter workers (33) inferred from other results in their work that strains "C," "D," and "E" were more closely related to strain "B" than to strain "A" and that strain "G" was more closely related to strain "A" than to strain "B." This opinion was based upon the observation that strain "A" almost invariably developed as the first relapse strain following initial attacks with strains "C," "D," or "E," while initial infections with strain "G" almost invariably led to the appearance of strain "B" in the first relapse.

Paramecium Variation

Harrison & Fowler (35) considered certain factors related to the phenomenon of antigenic variation in a study of the free-living, ciliated protozoan, *Paramecium aurelia*. Three methods were employed for the isolation of variant substrains. These were: (*a*) growth of mass populations in media containing antiserum in concentration effective against one or more of the variant forms in the population; (*b*) growth of mass populations from individuals selected at random without regard to their antigenic character; and (*c*) growth of mass populations from individuals whose antigenic character was determined by serologic test. No difference was found in the substrains isolated by each of these methods from any given parent stem strain.

A point of further interest in the work was the demonstration that the character of subcultures was apt to reflect the over-all antigenic character of the parent stem culture, irrespective of the character of the individual cell from which it was propagated. On numerous occasions it was found that cultures entirely similar to the parent culture in over-all antigenic make-up were cultivated from one of the few individuals in the parent clone which was found in direct test to be antigenically at odds with the majority of the population. For example, when 250 individuals from culture "A" were tested in antiserum "C" five animals were found insensitive and 245 were found sensitive. One of the insensitive individuals grew, upon isolation and propagation, into a clone in which nearly all of the individuals were sensitive to serum "C." On some occasions the sensitivity of the derived clone was greater than the

parent clone, even though the derived clone was developed from an insensitive individual. For example, parent culture "P" was composed of equal numbers of individuals sensitive and insensitive to serum "30" in highest dilution of 1-32. One of the insensitive individuals developed, upon isolation and propagation into a clone which, at the time of testing, was homogeneously sensitive to serum "30" in dilution of 1-1024. In still other instances and by the same methods, it was shown that variant cultures might be developed from individuals which, in direct test, seemed not themselves to be variant forms.

Another finding in this work with the paramecia may be of interest. One of the stock cultures which had shown variability between daughter subcultures and individual progenitor cells such as that related in the preceding paragraph was subjected to further study at a time when about 4 per cent of the individuals in the culture were insensitive to serum "54" while the remaining 96 per cent were sensitive. Among seventy-eight clones established from individuals selected at random from the parent culture seventy-five, or 86.1 per cent, were sensitive to serum "54" while three, or 3.8 per cent, were insensitive. Although no attempt was made to characterize each individual progenitor in this experiment, the author is quite doubtful that each of the individual progenitors were antigenically homologous with their daughter clones. It should be pointed out here that a divergence in character of parent and the mass of individuals in the population developed by asexual processes from it, particularly when the parent itself is divergent from the mass of its own population, is not an unusual occurrence in the biology of protozoa. Jennings (36) cites a number of experiments with *Difflugia corona* relating to the number and length of spinous processes in which the results are in a general way comparable to those secured in the study of antigenic variation with the paramecia.

Stability of Antigenic Character

It is quite evident, in spite of the great frequency and extensive range of antigenic variation reported for many species of microbes, that a striking degree of stability holds in the over-all antigenic pattern of pure cultures. In other words, some limits are operative in the frequency and extent of antigenic variation. There can be no doubt, however, that microbial populations, like populations

of multicellular organisms, reflect, through the course of many generations in series, one or another circumstance of the environment. It has been seen that bacterial and protozoan populations are sensitive to antibody in the environment to an extent at least which brings about a selective change in the per cent distribution of inherent antigenic types if not, indeed, to an extent which involves the appearance of entirely new antigenic variants. It has also been seen that the inclusion of substances other than antibody in the environment, such as organismal extractives or body fluids from mammals, may bring about a change in the character of the population. Nevertheless, various strains of protozoa and bacteria show different degrees of stability among their antigenically variant components even when the circumstances of environment seem to be constant.

The *Salmonella*, for example, show different degrees of stability among their antigenically variant forms. The usual strains of *Eberthella typhi*, are normally quite stable in antigenic composition of flagellar structures, and their variant *Beta* forms, which may be isolated only with difficulty, show the same high degree of stability. Kauffmann (17) considered the *Beta* variants of this species to be irreversible, while Edwards & Bruner (20) reported that only one of fourteen *Beta* variants could be reversed to the original form. These latter workers also noted that only two of sixty-five colonies selected at random from the reversible culture could be reversed. Contrary to the high degree of stability shown by *E. typhi*, in either the *Alpha* or *Beta* phase, other strains belonging immunologically to the same group, *Salmonella*, show little stability in the described antigenic variants. These unstable, diphasic strains constantly show two or more reversible variants during active subculture. Attention should be called to the fact, however, that these diphasic strains are quite stable in over-all character in that they normally do not show, with the usual tests, variants other than the two natural phases.

The work of Harrison (37) presents an interesting case of stability in immunologically variant forms of many strains of oral lactobacilli. Among thirteen strains the eleven which were fully tested in the work revealed a marked antigenic difference between the form prevailing at the time of isolation from human beings and the form prevailing after a long period of cultivation on laboratory media. This change involved the replacement of the type of ex-

tractible carbohydrate antigen in the early cultures with a different carbohydrate antigen in the late cultures. Six of these cultures were found to have undergone changes in metabolic activities and in morphologic character which were, however, not associated with ordinary smooth to rough variation. One of these cultures underwent a complete antigenic change of the capsular polysaccharide in a period of seventeen months. An intermediate form, derived and tested in the eleventh month of the study, reacted in anti serum for both the early and the late cultures. Since the study did not include examination of the early and late cultures for the presence of small numbers of variant cells it is not clear whether the cultures were antigenically homogeneous, although they were purified from time to time by colonial isolation. The author suggested that the change may have occurred as a result of selection or of environmental modifications or perhaps from the development of new activity by adaptive enzymes. In any event, the cultures did exhibit marked stability in over-all antigenic character in one form for a long period, then changed to another form.

Cunningham, Theodore & Fraser (33) noted that, while nearly all of their cultures of *Borrelia carteri* remained unchanged during prolonged and rapid subpassage through as many as five hundred animals, a few of them tended to revert to the more stable forms "A" and "B" during rapid subpassage in animals in which a detectable amount of antibody had not been produced. They reported twenty-eight such variations independent of antibody action, and in several of these cases the infecting brood of parasites was found to be composed of individuals of two or three antigenic types. Further, they found on several attempts that inocula purified by screening through selected antiserums *in vitro* developed the same and different antigenic variants during subsequent animal passage. Four such occasions of variations which developed apparently without antibody intervention are presented in diagrammatic form in Fig. 3. The view, indicated here, that the "A" and "B" variants were the more stable ones of *Borrelia carteri* is in agreement with other findings of these workers that the "A" and "B" variants were, by far, most frequently encountered during the first relapses in animals following recovery from the primary attacks initiated by strains "C," "D," "E," "F," "G," "H," and "I." This view also accords with and may serve to explain the fact, observed by Cunningham (31) and Cunningham & Fraser (38)

that strains "A," "B" and "C" were the ones most frequently found in human beings suffering from louse borne relapsing fever in India.

Stability of antigenic character in the trypanosomes appears

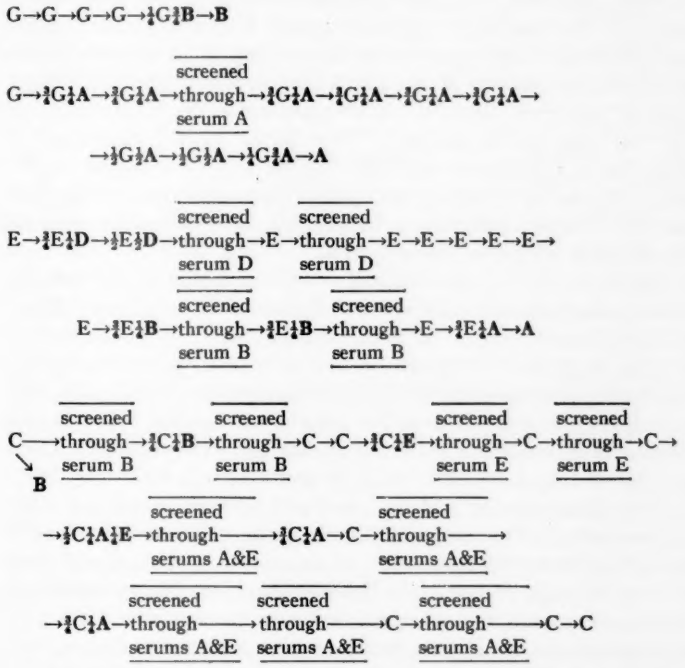

FIG. 3.—DIAGRAMS OF VARIATIONS IN ANTIGENIC PHASE OF SPIROCHETES OBSERVED BY CUNNINGHAM, THEODORE & FRASER (33) DURING RAPID SUBPASSAGE THROUGH SQUIRRELS WITHOUT INTERVENTION OF ANTIBODY PRODUCTION. FRACTIONS INDICATE PROPORTIONS OF EACH TYPE DEVELOPED DURING PHASE VARIATION.

to be quite similar to that in the *Borrelia*. These organisms have frequently been reported to show a striking degree of antigenic stability when maintained in mice without detectable antibody development [cf. Taliaferro (1)]. However, the number of instances in which relapse broods are found to be mixed would seem to indicate that these organisms are unstable in some antigenic phases. A number of workers have observed that as a rule one immunologic

type of trypanosome will occur much more frequently than others as the first relapse strain when animals are inoculated with the same parent strain. Raffel (39) concluded that each of the first relapse strains of *T. equiperdum* secured in three different rats were the same. Lourie & O'Connor (29) also noted that certain antigenic types of *T. rhodesiense* appeared more frequently than others during the first relapses in mice. Thus type "b" appeared in nine of twenty-two relapse strains, type "h" in five, type "a" in four, type "c" in three, type "d" in two and types "e," "f," "g," "i," "j," "k," and "p" in one each. These latter workers also reported that one in twenty-two of their relapse strains reverted to the antigenic variety of the parent strain during the course of a few months of rapid subpassage in animals in which there was no intervention of relapse phenomenon.

Harrison & Fowler (35) noted a marked difference in stability among a large number of strains of *Paramecium aurelia* and *Paramecium bursaria*. Some of these strains have been observed to undergo no noticeable change in over-all character of the population during the course of more than four years study in which they have been maintained in active growth continuously and tested frequently (part of this information has been unpublished hitherto). One strain, established as a variant strain in February 1943, was found to be in the variant form in all tests up to and including the one made in February 1947. Among nine other variant strains, established in a similar manner, two reverted to the original type in three months or less, two reverted between the seventh and fourteenth months, five remained in the variant state for seventeen months or more. Among the last five, four reverted to the original parent type while one has remained in the variant state throughout four years time during which it was transferred to new mediums 107 times. On the other hand the parent strains from which these variants were isolated have been observed to be quite unchanged in over-all antigenic character during four years of study.

It appears that among all genera of microorganisms in which the phenomenon of antigenic variation has been studied extensively, various species are more or less well stabilized in the long run, and under constant conditions, in a form in which all individuals are of the same antigenic type or in a form composed of individuals of different antigenic types in some state of equilibrium.

The tendency of organisms to revert from an over-all antigenically variant form established in one way or another to the original parent form when conditions are similar to those under which the parent originally was found or established is also quite apparent for many species though it is not at all apparent in a few cases (*E. typhi*, *Beta* variants for example). These aspects would seem to indicate that some inherent stabilizing force remains within lines of propagation of the organism though it may not be apparent for considerable periods of time.

Discussion

There can be no doubt that the examples of antigenic variation already reviewed are instances of inheritable change. However, it is not entirely clear whether they represent genetic mutation or nongenetic modification. Nor is it clear whether the changes in various examples of variation are due to a selection of variant individuals which arise spontaneously or to some adaptation of individuals in the population to circumstances of environment.

It should be pointed out, with regard to the question of mutation or modification, that in none of the examples reviewed is the variation a temporary modification such as that reported by Robertson (40). This worker observed that individual cells in cultures of the free-living, protozoan group, *Colpidium-Glaucoma*, lose sensitivity to homologous antibody following initial reaction and retain an insensitive condition so long as a suitable concentration of antiserum is provided in the culture fluid. Shortly after the insensitive individuals are placed in medium which does not contain antiserum they regain sensitivity to the antibody. These findings have been confirmed in the preliminary report of Harrison & Fowler (41) and in subsequent unpublished work by the same investigators. On the other hand the instances of variation described in previous sections of the present article have, with the exception noted, been changes which last in the populations through several generations.

The frequency of antigenic variation, so far as it is known, the range of diversity in antigenic type in given species and the stability of variant forms suggest that these changes are mutations in the broader meaning of the term. Bornstein (42) asserts that when a subculture of a young single colony of a diphasic *Salmonella* strain is incubated in broth all of the flagella in the culture are

antigenically identical for about six hours. If this is true it is evident that the variant cells do not appear until the population has reached at least the number of 10^6. Such rate of change falls well within the rate of gene mutation indicated for corn by Snyder (43) who presents mutability of several genes in the following frequency per million: "R," 492.0; "I," 106.0; "Pr," 11.0; "Su," 2.4; "Y," 2.2; "Sh," 1.2; "Wx," 0.0. The studies of Harrison & Fowler (35) on the rate of change in antigenic character in populations of *Paramecium aurelia*, though inconclusive in populations numbering less than 100,000 or more progeny from a single parent, indicate that the variant form does not appear in frequency much greater than that cited for corn.

The extent of antigenic variation observed in most species of microorganisms and its cyclic nature in some, relates well to the theory of mutational change expressed by Muller (44) as follows:

"However, a defensible case for the existence of separable genetic material might have been made out on very general considerations alone. Despite the baffllingly complex and seemingly erratic character of biological things, and their change at the touch of analysis—characteristics which have made them such a happy hunting ground for obscurantists—it is obvious that the whole congeries of variable processes of each kind of organism tends to go in a succession of great cycles, or generations, or even alternations of generations, with the generations in turn made up of smaller or cell cycles, and that at the end of every greatest cycle something very like the starting-point is reached again."

Permanency, in some degree or other, was taken as a point of difference between bacterial modification and mutation by Dobell (45). There can be no doubt that the over-all antigenic characters of bacterial populations are, if environmental circumstances remain constant, decidedly stable.

These changes then seem to indicate that the agency of antigenic control in bacterial organisms is quite analogous to the agencies which control stable characters in higher organisms. There is, however, but little evidence secured in direct observations on microbial populations which is informative on the chemical or physical nature of the agency with the exception of the results secured in studies of the pneumococcus.

In the case of the *Pneumococcus* it is quite apparent that an extractable agency, partially definable in chemical terms, exerts a rigorous control on the antigenic type of the organism. Avery, MacLeod & McCarty (7) emphasize the fact that the changes induced by the transforming agency are not temporary modifications

but are permanent alterations which persist provided the organism is maintained under suitable culture conditions. McCarty (11) also emphasizes the fact that the agent, once it becomes a part of the cell, is reduplicated indefinitely with growth of the cell. Moreover, it should be made clear, that nowhere does it appear that pneumococci which have been transformed in type are different in any way in their new types from those of the same type which are isolated from nature. Dobzhansky (46) has expressed the view that the

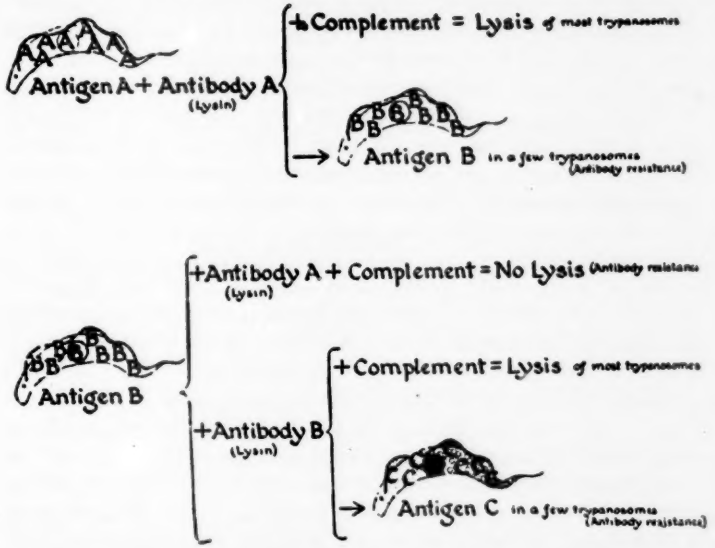
Antigen A + Antibody A
(Lysin)
+ Complement = Lysis of most trypanosomes
Antigen B in a few trypanosomes
(Antibody resistance)
+ Antibody A + Complement = No Lysis (Antibody resistance)
(Lysin)
Antigen B
+ Complement = Lysis of most trypanosomes
+ Antibody B
(Lysin)
Antigen C in a few trypanosomes
(Antibody resistance)

FIG. 4. DIAGRAM OF THE POSSIBLE RELATIONSHIP OF ANTIGENIC CHANGES TO ANTIBODY RESISTANCE OF THE PATHOGENIC TRYPANOSOMES. EACH LYSIN WHEN PRODUCED BY THE HOST KILLS A MAJORITY OF THE HOMOLOGOUS TRYPANOSOMES, BUT A FEW OF THE TRYPANOSOMES ESCAPE ITS ACTION BY BECOMING CHANGED ANTIGENETICALLY [TALIAFERRO (47)].

transformations in pneumococcus types by vaccines "represents authentic cases of induction of mutation by specific treatments." Muller (44) expressed the view that the most probable explanation of these transformations is that of entrance of foreign genetic material into the control system already there by a process "essentially of the type of the crossing-over process" described for metazoan organisms.

From other points of view it is nearly as clear that some agency exerts rigorous control over antigenic character of other species of microorganisms. This agency, as has already been pointed out, is apparently subject to mutation. Its sensitivity to environmental factors, however, is not clear. The concept of organismal sensitivity to antibody action has at times leaned strongly to the notion that specific change, either a mutation or a more or less permanent modification, is induced by the action of antibody on the control agency. The response of trypanosomes to the action of antibody particularly has been interpreted by many investigators [cf. Taliaferro (1)] in this way. Thus Ehrlich postulated a change in character of nutriceptors by the action of antibody—the new nutriceptor in turn brought a change in the antigenic character of the organism. A diagrammatic representation of this concept is presented in Fig. 4. This concept also has been used in explanation of antigenic variation in the *Borrelias*. Cunningham, Theodore & Fraser (33) were of the opinion that antibody induced a change in type of *Borrelia carteri* in spite of the fact that change was found to occur in twenty-eight instances in which antibody could not be demonstrated. However, it should be pointed out that the strong selective action of antibody which is very evident, could, in operation with spontaneous mutation, account for all of these results. In extensive studies of variation in *Paramecium aurelia*, Harrison & Fowler (35) were unable to induce, by antibody action, modifications beyond those which were found to occur normally.

Taliaferro (47) has pointed out that whatever underlying mechanism is involved in the variations reported for the trypanosomes it is probably the same as when free-living species become acclimatized to various chemicals. It also seems that the antigenic variations in the bacteria are in general quite analogous to the acclimatization of bacteria to a variety of unfavorable chemical agents. Acclimatization of protozoa to toxic chemicals has been attributed to an adaptive process, called *Dauermodificationen*, by Jollos [cf. Jennings (36)]. On the other hand the acclimatization of bacterial populations to penicillin, to streptomycin and to a variety of mercuric and copper salts has been attributed to selection of spontaneous mutants by Demerec (48), Klein & Kimmelman (49) and Severens & Tanner (50) respectively.

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LIFE CYCLE OF MALARIAL PARASITES

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The deficiencies in knowledge of the life cycles of the malarial parasites of man were given emphasis by the great importance of this disease in World War II and by the urgency for bringing it under control. This unsatisfactory state of knowledge extends also to the malarial parasites of animals. In most instances in which the transmitting host is known those phases of the life-cycle of the parasite occurring in it have been more completely studied than the stages in the vertebrate host. However, the transmitting hosts are still unknown for all chiropteran and reptilian species of *Plasmodium*, for the reptilian species of *Haemoproteus*, for several species of simian *Plasmodium* and for many avian species of *Plasmodium*, *Haemoproteus*, and *Leucocytozoon*. During the past two decades great interest has centered in the phases of the life-cycle which develop in the vertebrate host, especially that during the prepatent period. This review will be concerned with an evaluation of much of the work which has been published on exoerythrocytic schizogony and particularly on the pre-erythrocytic stages. Reviews of the literature on exoerythrocytic schizogony have been made by Verney (102) Porter & Huff (76) and Kikuth & Mudrow (57), and on the pre-erythrocytic stages by Huff & Coulston (41) and Huff (39).

Following the discovery by Laveran (58) and by Danilewsky (25) of malarial parasites in the blood of man and of birds respectively, knowledge of the erythrocytic stages of these parasites grew rapidly. The parasites were readily obtainable in the peripheral blood and methods for staining and study of them were soon widely known. Likewise the cycle in the mosquito was readily worked out as soon as Ross (88) and Grassi, Bignami & Bastianelli (36) had discovered the vectors of avian and human malaria respectively. Grassi (35) described and depicted these stages in the mosquito so accurately that subsequent research on them has yielded little

* Grateful acknowledgment is made to Mr. J. T. Maloney for translating some of the Italian papers.

of significance that is new. There was speculation by some of the early writers on malariology, particularly Golgi (33) and Grassi (34) on the possibility of the development of the parasites in locations other than the red corpuscles. However, Schaudinn (90) described and illustrated the entry of sporozoites into erythrocytes so convincingly as to set the pattern of thinking on this phase of the life cycle for more than a score of years. Textbooks during that period uniformly indicated a completed cycle with the sporozoite entering directly into erythrocytes.

The indirect evidence which led to the questioning and overthrow of Schaudinn's explanation of the beginning of parasitism in the vertebrate came in four different lines; namely (*a*) the chemotherapeutic evidence; (*b*) failure to confirm Schaudinn's observations; (*c*) the discovery of the non-infectivity of the blood during the incubation period; and (*d*) the discovery of actual exoerythrocytic stages in certain avian species of *Plasmodium*.

The Sergent brothers (95) first noticed the differences in the efficacy of quinine against avian malaria when given therapeutically as compared to its prophylactic administration. The greater efficacy of quinine against blood-induced *P. vivax* infections than against sporozoite-induced infections was observed by Yorke & MacFie (107) and this was amply confirmed by James (47), Swellengrebel & de Buck (98), Russell & Nono (89), Kikuth & Giovannola (52) and Missiroli (66) on several other species of malarial parasites. The intensive investigation involving thousands of drugs and many different species of malarial parasites carried out during and since World War II have served only to confirm this evidence. Although too extensive to be covered by this review we shall return later to a discussion of this evidence in relation to the possible existence of exoerythrocytic stages in human malaria. Suffice it to say here that the chemotherapeutic evidence favors the existence of stages of development between the sporozoite and the erythrocytic trophozoite.

Several investigators failed completely to confirm Schaudinn's observations that sporozoites may enter erythrocytes directly. These include Yorke & MacFie (107), Yorke (106), Boyd & Stratman-Thomas (8), and Raffaele (79) and other unpublished negative attempts [see Huff & Coulston (41)].

The existence of a "negative phase" or period of infection without parasitemia was found by Boyd & Stratman-Thomas (8),

Kikuth (51), Warren & Coggeshall (103), Kikuth & Mudrow (54), Raffaele (81), Missiroli (66), Ciuca *et al.* (11), Boyd & Matthews (7), Henry (37), Coulston, Cantrell & Huff (19) and Fairley (28) in several species of parasites. In avian infections Warren & Coggeshall (103), Kikuth & Mudrow (55, 56), de Court & Schneider (27) and Coulston, Cantrell & Huff (19) proved that infection exists during the stage when the blood lacks parasites by sacrificing the host during this stage and implanting various tissues in uninfected animals.

Parasites which were actual exoerythrocytic stages were observed and figured by Anschütz (1, 2) and BenHarel (3). The species with which they were dealing were probably *P. circumflexum* and *P. elongatum* respectively. The true significance of their observations was not recognized at the time. The abundance of *P. elongatum* in red cell precursors of the hemopoietic tissues was observed by Huff (38) and Raffaele (79). The latter called attention to the absence of pigment in many of these stages and later he (80) observed that they parasitized a wide variety of cells, some of which he wrongly placed in the reticulo-endothelial system. Huff & Bloom (40) described and portrayed schizogonic forms of *P. elongatum* in all cells of the lymphoid and myeloid series including lymphocytes, monocytes, plasma cells, thrombocytes, macrophages, hemocytoblasts, erythroblasts, erythrocytes, and myelocytes. They indicated the synchronism of schizogony in the parasites of the blood and in those of the bone marrow. Exoerythrocytic stages showing a preference for endothelial and reticulo-endothelial cells were demonstrated by Raffaele (82) in *P. relictum*, by James & Tate (49) in *P. gallinaceum*, by Kikuth & Mudrow (53) in *P. cathemerium*, by Coulston (18) and Manwell & Goldstein (61, 62) in *P. circumflexum*, and Purchase (77) in *P. durae*. Porter (75) classified the exoerythrocytic stages occurring in birds as (*a*) elongatum-type and (*b*) gallinaceum-type depending upon whether they prefer erythroblasts and hemocytoblasts or macrophages and endothelial cells respectively. Thompson & Huff (101) described exoerythrocytic stages of both of these types in lizards infected with *P. mexicanum*.

Terminology

Although the term "exoerythrocytic stage" was proposed by James & Tate (49) for the large unpigmented schizonts which they

found in infections with *P. gallinaceum*, the term was extended to the stages of *P. elongatum* which live predominantly in the erythroblastic series of cells, and has since come to include all stages of *Plasmodium* which undergo any considerable portion of their development in cells other than erythrocytes [Porter & Huff, (76)]. Authors writing both in English and German have often abbreviated the term to E-E stage. Since exoerythrocytic stages occur even in blood-induced infections with some species of *Plasmodium* a distinction must be made between such stages and those derived from sporozoites. For the first generation of parasites developing from the sporozoite Huff, Coulston, & Cantrell (44) proposed the term cryptozoite. Subsequent generations occurring prior to the establishment of parasitemia were called metacryptozoites by Huff & Coulston (41). The term pre-erythrocytic stages has been employed as a collective designation for all stages between the sporozoite and the erythrocytic stage. For the remaining category of stages—the exoerythrocytic stages exclusive of pre-erythrocytic ones—Huff & Coulston (42) proposed the term, phanerozoite. Although not universally adopted in its entirety this nomenclature will be used here for the sake of clarity.

Pre-erythrocytic Stages in Avian Malarial Parasites

Even though the indirect evidence cited above was valuable in the formation of an hypothesis that sporozoites need to undergo development elsewhere than in erythrocytes before the beginning of parasitemia, observations of stages in this development needed to be made before the hypothesis would be considered as proved. Observations of exoerythrocytic stages in blood-induced infections, or after parasitemia had been established in sporozoite-induced infections, made the hypothesis more plausible but did not necessarily prove it. Strains of avian malaria in which exoerythrocytic stages could be found were capable of being passed through many blood transfers and still exhibit these stages. Also Coulston & Manwell (22) demonstrated that exoerythrocytic stages could arise from erythrocytic stages through their experiments in which clones of *P. circumflexum* were established from erythrocytic stages.

Attempts were made by Missiroli (64 to 69) to observe the transitional stages from sporozoites to erythrocytic forms in *P. relictum*. He claimed as a result of his experiments that, after ten minutes in the canary, sporozoites lose their cytoplasm and the re-

maining nuclei then behave essentially as sporocysts. He based this belief upon the observation of 2, 4, 6, and 8 granules in the nuclei which he interpreted as steps in transformation from sporocyst to sporozoites. He could not demonstrate developing stages in the inoculation site (muscle) or in the spleen, liver, lung, or bone marrow of birds inoculated with sporozoites. He concluded that sporozoites could not complete their development at the inoculation site and believed (68) that they enter neither erythrocytes nor reticuloendothelial cells but develop extracellularly. He (69) claimed that between 12 and 30 hours these extracellular, 4-nucleated forms develop and divide into four merozoites which then enter erythrocytes. Schulemann (91) also claimed a somewhat similar kind of development for this species.

Kikuth & Mudrow (56, 57) made observations on the inoculation sites and internal organs of canaries inoculated with sporozoites of *P. cathemerium*. The stages which they saw in touch preparations of the site of inoculation in the pectoral muscle after 16 hours were predominately uninucleate and some, but not all, of them were intracellular. At 40 hours the forms they saw were chiefly intracellular and uninucleate, and after 48 hours some were multinucleate, a 5-nucleated one being the latest stage of development seen. At 64 hours sparse multinucleate forms were seen.

Schulemann & Spies (92) attempted to follow the development of sporozoites of *P. gallinaceum* in the skin of chickens in which sterile sponges had been implanted eight to ten days earlier. They described from sections made of the local area 24 hours after the injection of sporozoites 4-nucleated stages, some intracellular, some extracellular. Larger forms with many nuclei were found between 40 and 65 hours. They identified the host cells as histiocytes.

Reichenow & Mudrow (87) described the early development of *P. praecox* (=*relictum*) in canaries inoculated with sporozoites. Their observations were made from 13.5 to 189 hours after inoculation. The stages seen at 13.5 hours were unincleate and those at 24 hours 2- to 4-nucleate. At 35 hours they observed parasites with 16 and 24 nuclei and at 40.5 hours a segmenter with forty merozoites and several unincleate forms.

Paraense (72) observed sparse pre-erythrocytic stages of *P. gallinaceum* within the first 24 hours after sporozoite inoculation and more frequently at 60 and 84 hours. His preparations were made from scrapings of the under surface of the dermis.

Huff & Coulston (41) described and illustrated the complete development of *P. gallinaceum* from the entrance of sporozoites into the skin of chickens to the appearance of erythrocytic stages in the blood. With the exception of Schulemann & Spies (92) they were the only investigators who used material which had been fixed, sectioned and stained in a manner permitting the identification of the host cells and a study of the exact relationship between the parasites and the cells of the host. They compared their findings made in this way with parallel studies employing the ordinary technics of smears stained with Romanowsky's stain. Sporozoites inoculated either naturally by mosquitoes or artificially by hypodermic needle were taken up by phagocytic cells in 0.5 to 6 hours' time. Those engulfed by heterophil leucocytes were either killed as sporozoites or early in their development within these cells. However, those engulfed by cells of the lymphoid-macrophage system by the end of 6 hours lost their sporozoite appearance and began to grow rapidly. They assumed a spherical or oval shape and grew in size for the first 36 hours and during this time there was rapid division in the nuclei. Segmentation was completed between the 36th and 48th hours. The numbers of merozoites were difficult to count accurately in sections but were probably 75 to 150 per segmenter. The merozoites entered new lymphoid-macrophage cells and also endothelial cells of the blood vessels and underwent development similar to the first generation. After three or four such generations erythrocytic parasites began to appear. The earliest that an erythrocytic stage was seen was at 75 hours but this appeared in the blood of a small chick which had been inoculated with the sporozoites from three hundred infected *Aëdes aegypti*. They appeared between the fifth and tenth days in animals which had received moderate numbers of sporozoites.

That some sporozoites develop at the site of mosquito bite was shown by Kikuth & Mudrow (56) for *P. cathemerium* and by Huff & Coulston (41) for *P. gallinaceum*. The experiments of Coulston, Cantrell, and Huff (19) on localization of sporozoites following mosquito bite indicated that some of the sporozoites are transported (probably by the blood) rapidly to the spleen. When large numbers of sporozoites were injected intravenously they were able to develop for the first generation in kidney, muscle, spleen, pancreas, lung and liver. The second generation could develop in all of the organs tested (19, 41).

The relationship of the pre-erythrocytic stages of *P. gallinaceum* and *P. relictum* to the natural and acquired immunities of various hosts was reported by Huff & Coulston (42). They showed that both natural and acquired immunity were less effective in preventing growth of the pre-erythrocytic than of the erythrocytic stages. Thus when ducks were inoculated with sporozoites of *P. gallinaceum* the pre-erythrocytic stages grew well in the tissues but no parasitemia was produced. Subpatent infections persisted in some individual ducks for as long as eight months after they had been inoculated with sporozoites but it appears probable that these infections were continuations of the tissue stages. Pre-erythrocytic stages grew in hyperimmune animals which had received repeated inoculations of both sporozoites and erythrocytic stages.

By methods similar to those used by Huff & Coulston (41) the pre-erythrocytic stages of *P. relictum* were studied by Coulston & Huff (20) and of *P. lophurae* by Huff *et al.* (45). The essentials in the development of these two species, together with that of *P. cathemerium* [Huff & Coulston (43)] are similar to those of *P. gallinaceum*. Morphologically the pre-erythrocytic stages of *P. gallinaceum* are very similar to those of *P. lophurae* while those of *P. cathemerium* are similar to those of *P. relictum*. The pre-erythrocytic stages of *P. lophurae* were observed in turkeys, ducks, chickens, and guinea fowl [Huff *el al.* (45)]. In addition to those which were normal in appearance abnormal forms were observed in all four hosts. There was no apparent correlation between the degree of parasitemia produced by sporozoite inoculations and the degree of abnormality of the pre-erythrocytic stages.

It is, therefore, clear then that pre-erythrocytic stages not only exist but are a necessary part of the life-cycle of at least four species of avian malaria. The findings of Kikuth & Mudrow (56), Reichenow & Mudrow (87), Huff & Coulston (41, 42), Coulston & Huff (21) and Huff *et al.* (45) invalidate the claims of Missiroli (cited above) about the sporocystic nature of the sporozoite, the loss of its cytoplasm and their development extracellularly. All four species in which the pre-erythrocytic stages are known have the ability to produce exoerythrocytic stages (phanerozoites) of the gallinaceum-type even when passed from bird to bird by inoculation of infected blood. As yet, the behavior of sporozoites of *P. elongatum*, which exhibits phanerozoites strikingly different from the four species just discussed, has not been observed. This is true also of all the

species of avian *Plasmodium* in which no exoerythrocytic stages have been found. Likewise this phase has not been studied in any of the species of saurian *Plasmodium*.

Evidence Concerning the Existence of Exoerythrocytic Stages in Human and Simian Malarial Parasites

The situation in regard to the possible presence of either preerythrocytic or phanerozoitic stages in the malarial parasites of mammals is by no means as clearly defined as that just described for avian malaria. There are several claims that such stages have been observed in simian and human malarial infections. There are, on the other hand, several records of inability to discover such stages and a considerable reluctance on the part of many malariologists to consider the problem to be completely resolved by the evidence so far adduced. The number of publications giving negative results is small but this does not reflect the true situation since there is always more reluctance to publish negative results than to make positive claims.

Schwetz (93) observed in the bone marrow of a *Cercopithecus* monkey infected with *P. kochi* some unpigmented bodies with fifteen to thirty structures which might be interpreted as nuclei. He pointed out their resemblance to agamonts of *Theileria* but made no conjecture as to their real nature. In 1938, after exoerythrocytic stages had been reported by others in species of avian *Plasmodium*, Schwetz (94) in a letter to the Royal Society of Tropical Medicine and Hygiene indicated that he was convinced that the bodies which he had previously described were analogous to those found in avian species of *Plasmodium*. It is worth noting that Schwetz's original findings were made after the death of the animal and that his claim that the structures in question were exoerythrocytic stages of malaria was made five years later without further study of *P. kochi* in monkeys.

Raffaele (83) described from smears made of the sternal marrow of patients inoculated with sporozoites of *P. vivax* one nonpigmented rosette and two trophozoites without pigment in a "histiocytic reticular cell." In 1938 this same author (84) described one unpigmented stage of *P. falciparum* in smears of sternal marrow made on the sixth day after inoculation. Raffaele (85) portrayed another schizont from the bone marrow of a patient infected with *P. falciparum* and, in addition, two supposed exoerythrocytic

stages from a patient infected with *P. malariae*. Tarsitano & Lucrezi (100) also reported exoerythrocytic schizonts from the bone marrow of a patient infected with *P. vivax*. Brug (9) described three exoerythrocytic stages of *P. vivax* from lung smears of a corpse forty-eight hours after death. The patient had died ten days after an intravenous inoculation of infected blood. The peripheral blood was infected at the time of death. It is worthy of note that at the time Brug's preparations were made even many of the host cells were degenerating. His figures bear little resemblance to any known stages of malarial parasites. Bianchi (4) has reported exoerythrocytic stages in the bone marrow of a patient infected with malignant tertian malaria and Garcia (32) described such stages of *P. vivax* and *P. falciparum* from the brains of persons dying from their infections. Coudert & Eyquem (17) described unpigmented stages from blood-induced infections with *P. vivax*. These bodies measured 2μ by 3μ and sometimes as many as ten of them were in one host cell. Because of their sharpness of contour and the character of their staining they were thought not to be parasites which had been freed from their host cells and then phagocytosed. They also made inoculations from the bone marrow of a patient during the incubation period and claimed to have transmitted infection at a time when the blood was negative. Examination of the bone-marrow revealed the presence of tissue stages at the time of transfer and before the blood became infected. The claim that exoerythrocytic stages were readily found during the prepatent period in blood-induced infections is contrary to the situation in avian malaria in which exoerythrocytic stages do not become abundant until a time shortly after the peak of parasitemia has been attained. Matilla & Apariico (63) found extracellular forms of parasites in the bone marrow of malarious patients (details of infections and patients not given) and commented on the presence of pigmented as well as of unpigmented forms. It is not surprising that making smears from bone-marrow should produce artifacts. Oberlé (71) also studied smears from sternal punctures of patients with blood-induced infections with *P. vivax*. In all of his preparations he found only one stage which could be likened to the exoerythrocytic stages described by others, but he recorded the presence of numerous extracellular forms in the smears from marrow made on the fifth day after inoculation. Fonseca, *el al.* (31) reported upon observations made on smears from sporozoite-induced

infections of both *P. vivax* and *P. Falciparum*. Autopsies were performed between six and twenty-four hours after death and smears were made from spleen, liver, brain, bone-marrow, and lungs. No positive findings were made in the smears from patients dying twenty-four and forty-eight hours after inoculation. In the smears from two patients with benign tertian malaria dying five and eight days after inoculation they reported finding intra- and extracellular bodies resembling exoerythrocytic stages of avian malaria. In one case of malignant tertian they found forms in the brain after eight days one of which they considered as a "probable extracellular form." They believed they also saw parasites of this species in cells of the capillary walls. The objects portrayed in their colored plate resemble many of the artifacts commonly seen in tissue smears. Casini (10), Paraense & da Silva (73), and Garcia (32) have, in addition to the last mentioned authors above, described in the brain capillaries of patients dying with *P. falciparum* infections, extracellular, unpigmented parasites and stages appearing to be in the cytoplasm of the capillary endothelial cells, and have likened those to the exoerythrocytic stages known in avian malaria. From their descriptions and illustrations it is difficult to see how the structures described by them differ essentially from the capillary localizations so well known in the pathology of malignant tertian malaria. The writer has been demonstrating similar material to his classes for over fifteen years to illustrate the well-known capacity of *P. falciparum* to localize in capillaries of various organs. It seems significant to quote from James' (46) section on the pathology of malaria in Byam & Archibald's classic textbook:

> "In comatose cases which have ended fatally it is usual to find that the capillaries and small arterioles are blocked with numerous red cells containing parasites often in the sporulating stage. Parasites apparently not in red blood cells are also found, and numerous pigmented leucocytes, macrophages and endothelial cells as well as much free malarial pigment. The parasite-infested corpuscles have a tendency to cling to the sides of the vessels and in severe cases there is complete occlusion and thrombosis."

For further references to this phase of the pathology of malaria, pages 44 to 46 of Taliaferro & Mulligan (99) may be consulted.

Several investigators have recorded negative findings in their search for exoerythrocytic stages of human malaria. Sicault & Messerlin (96) stated that they had been searching systematically

by splenic and sternal puncture since 1934 for these stages. They did not indicate the species of parasite involved. They stated that it was exceptional to find schizonts in these organs when they have disappeared from the blood, and they made no statement indicating that such schizonts were exoerythrocytic. Quattrin (78) examined the sternal marrow of sixty-one patients with sporozoite-induced infections of *P. vivax* during the prepatent period without finding a single exoerythrocytic stage. Parise & Lucreizi (74) examined by splenic puncture, and in some cases by sternal and hepatic puncture, seventy-six cases of chronic, naturally contracted malaria. Although they found hyperactivity of the reticulo-endothelial system they were unable to identify with certainty the existence of free or unpigmented stages of the parasites. Lioia (59) found no exoerythrocytic stages in the marrow smears of twenty-five cases of chronic malaria. Nucciotti (70) examined the marrow in thirteen patients with primary malaria. In two of them the findings were negative; in the other eleven all malarial parasites found could be identified as erythrocytic. The writer personally knows of many unpublished negative findings in research on simian and human malaria. Among these are the experiments of Huff & Coulston (43) which represent an extensive series of studies on human biopsied material taken from the site of inoculation of salivary glands from thirty or more *Anopheles quadrimaculaties* infected with *P. vivax*. These investigators also injected the sporozoites of *P. vivax* into the organs of five species of monkeys and the sporozoites of *P. cynomolgi* into the organs of rhesus monkeys and biopsied the sites of injection. The technics used were similar to those which were successful in demonstrating the pre-erythrocytic stages of *P. gallinaceum*, *P. cathemerium*, *P. relictum*, and *P. lophurae*, yet the results were uniformly negative.

The accumulation of negative results cannot be considered as a refutation of the positive claims for the finding of exoerythrocytic stages in mammalian malaria, but it does justify a re-examination of those claims to determine whether they can be accepted as proof of the existence of such stages. One or more of the following objections can be made to the claims for the discovery of these stages: (*a*) the examinations were made *post mortem;* (*b*) the technic used was one which contributed artifacts; (*c*) the stages reported were artifacts or erythrocytic stages which had suffered distortion; (*d*) the similarity with exoerythrocytic stages in avian malaria was

slight; or (*e*) the capillary localizations of *P. falciparum* were mistaken for exoerythrocytic stages. All studies made on autopsied material are especially subject to question since in some instances, such as in the article by Brug (9), degeneration was observable even in the host cells. In all of the reports of exoerythrocytic stages of malaria in man which were based upon material obtained from the living patient the method of study was by tissue smears or tissue impressions. This method which is so successful for blood is highly unsatisfactory for organized tissues. Even when wet-fixed with reagents adapted to quick fixation these preparations contain many sorts of distorted and disorganized cells impossible to identify. However, many of the studies were made on smears which were allowed to dry and were otherwise treated as blood smears would have been treated. Such preparations contain very little that can be identified with certainty and it is always possible to find artifacts in them which might bear some resemblance to exoerythrocytic stages.

The possibility that some of the so-called exoerythrocytic stages of *P. falciparum* may have been erythrocytic stages which had localized in the capillaries has already been noted. In this connection it should be added that free merozoites are often found in the clogged capillaries in which the localization has taken place and that, strictly speaking, they are exoerythrocytic and unpigmented but to call them exoerythrocytic stages in the accepted sense of the latter term would simply be terminological confusion.

Aside from the objections which may be raised individually to the claims for the finding of exoerythrocytic stages in human malaria it is clear that no real consistency exists among these claims in regard to type of cell or organ inhabited or in regard to their morphology and staining. Any cycle constructed from the published descriptions would be bizarre to say the least, whereas in avian malaria agreement was rather easily attained on the general features of the morphology of the exoerythrocytic stages soon after they were discovered.

Although many authors have accepted the claims for finding exoerythrocytic stages in human malaria there have been noteworthy exceptions. Among those who have expressed doubts about the validity of individual claims or about the collective claims for the demonstration of such forms may be cited: Reichenow (86), Wenyon (104), Corradetti (15, 16), Lioia (59), Fairley (29), and Davey (26).

In spite of the doubt which may be cast upon the published claims for the microscopical demonstration of exoerythrocytic stages in human malaria the indirect evidence for their existence from subinoculation experiments and from chemotherapy have been strengthened in recent years. The earlier experiments indicating the existence of a "negative phase" of the blood during the prepatent period in the infection of patients inoculated with sporozoites have already been cited. A more painstaking series of experiments was reported by Fairley (28) who transferred at least 200 ml. and sometimes as much as 500 ml. of blood from patients following the bites of heavily infected mosquitoes to test subjects. Positive results were obtained when the blood was drawn from patients within thirty minutes of the mosquito biting. However, after that period of time all results were negative until the seventh day in infections with *P. falciparum* or until the ninth day in infections with *P. vivax*. The evidence then is very conclusive that wherever the sporozoites and the subsequent three or four generations of schizogony occur it is not in the peripheral blood. Similarly, Cooper, Ruhe, & Coatney (14) have shown that in St. Elizabeth strain of *P. vivax* the blood is not infectious in the period of latency in patients who later suffered a relapse of their infections. They transferred 250 ml. of blood from six patients 181 days after their primary infection to six other persons. Only one of the six thus inoculated developed malaria and in this case the donor of the blood suffered a relapse seven days after he was bled for subinoculation. The other five recipients were found to be susceptible by testing them with intravenous injection of parasites on the 103rd day. Malaria failed to develop also in four persons inoculated with 150 ml. of blood 193 and 194 days after the primary attack in the donors. We may, therefore, conclude that the parasites (in the case of *P. vivax* at least) are not in the peripheral blood during latency. While we may not conclude that these experiments prove the existence of pre-erythrocytic or phanerozoic stages of human malaria the parallel with avian malaria in which such stages are known is very strong.

As already indicated, a review of the work done during the war on the chemotherapy of malaria falls outside the limits of this review. It is advisable, however, to review some of the work which bears upon the question of possible exoerythrocytic stages. The differences in efficacy of quinine when employed as a prophylactic and as a therapeutic agent which were earlier noted by the Ser ents

(95) and Yorke & MacFie (107) were shown by James, Nicol & Shute (48) and by Feldman *et al.* (30) to hold for pamaquin except in the reverse manner; that is, when given in large (toxic) doses during the first six days following sporozoite inoculation it served as a complete prophylactic against *P. falciparum* and greatly extended the prepatent period of *P. vivax* whereas its action against erythrocytic stages was weak. Other related drugs (8-aminoquinolins) were also shown to have prophylactic action by Loeb (60), and Jones *et al.* (50). A comparable action for proseptasine, a sulfonamide, against *P. falciparum* when the drug was given in large dosage was shown by Sinton, Hutton & Shute (97). Fairley (29) showed that paludrine, a drug differing in chemical structure from all previously mentioned ones, is a complete causal prophylactic in malignant tertian, a partial causal prophylactic in benign tertian malaria and has in addition, a powerful action against erythrocytic stages in both infections. Davey (26) has called attention to the fact that three chemically distinct compounds (8-aminoquinolins, sulfonamides, and paludrine) which show prophylactic action against human malaria are also known to have an action on exoerythrocytic stages of avian malaria. [See Coatney & Cooper (12), Coggeshall, Porter & Laird (13), Curd, Davey & Rose (24).] His implication is that this parallel action of the drugs suggests that pre-erythrocytic stages may be present in benign and malignant tertian malarias.

In sporozoite-induced infections of *P. vivax* Fairley (29) found that paludrine so cleared the blood of parasites that the subinoculation of 200 ml. of blood failed to produce infection in the recipients. However, overt malaria might later develop in the donor. He postulated that the drug inhibited schizogony in the hypothetical pre-erythrocytic stage.

Both Fairley (29) and Davey (26) state that the chemotherapeutic evidence favors more the existence of exoerythrocytic (phanerozoic) stages in *P. vivax* than in *P. falciparum*. They base this belief upon the greater ease of obtaining radical cures in malignant than in benign tertian malaria even when the drug used is quinacrine which is generally thought not to have any action against exoerythrocytic stages. However, Coulston & Huff (21) studied the effect of various drugs upon the cryptozoites of *P. gallinaceum* by cytological methods and found that quinine and quinacrine produced morphological changes comparable to those

produced by pamaquin or the sulfonamides which act as causal prophylactics. It seems somewhat unsafe to assume that there are qualitative differences in the behavior of any drug against the erythrocytic, pre-erythrocytic, and phanerozoic stages. It appears more likely that a drug produces—at a given concentration—somewhat similar effects although differing in degree on all stages of the parasite. The type of cell inhabited, the location in respect to the blood stream and possibly other factors might determine the degree of the harmful effect, and the order of these differences might vary with the kind of drug, the species of parasite, and the kind and condition of the host. Huff & Coulston (42) have shown the marked difference in the behavior of pre-erythrocytic stages versus the erythrocytic stages of *P. gallinaceum* in respect to the effects of both natural and acquired immunity. It would seem a reasonable hypothesis that in the case of *P. falciparum* infections any possible phanerozoic stages might be more readily killed than the pre-erythrocytic stages by a drug like quinacrine.

Since blood-induced infections of *P. cathemerium* and *P. gallinaceum* are as difficult to cure as sporozoite-induced infections whereas blood-induced infections of *P. vivax* are more easily cured by certain drugs than sporozoite-induced infections we might suspect that *P. vivax* does not pass as readily from erythrocytic to hypothetical exoerythrocytic stages as is the case with the avian parasites. If this were true we might expect that the numbers of sporozoites of *P. vivax* inoculated would appreciably affect the type of infection produced. The inverse relationship of dosage of sporozoites to prepatent period has been shown in *vivax* infections by James (47), Boyd & Kitchen (6), Boyd (5), and Whorton *et al.* (105). Boyd (5) also found a direct relationship between dosage of sporozoites and duration of clinical attack, while Craige *et al.* (23) found in the Chesson strain of *P. vivax* that the shorter the prepatent period, the earlier relapse may be expected and the less likelihood of an early cure. Taken together these findings may possibly indicate a relationship between numbers of sporozoites and (*a*) duration of clinical attack; (*b*) the earliness of relapse; and (*c*) the possibility of an early cure. There is a suggestion, though no clear proof, in these statements that any hypothetical phanerozoic stages in human malaria may arise principally if not wholly from the, as yet, hypothetical pre-erythrocytic stages.

Since the indirect evidence for the existence of exoerythrocytic

stages in mammalian malaria is good it would appear advisable to adopt their presence in sporozoite-induced infections as a working hypothesis. This hypothesis should, however, take into account the possibility that such stages may vary in important particulars not only from the known stages in avian infections but among the various species of *Plasmodium* occurring in mammals.

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VARIATION IN PHYTOPATHOGENIC FUNGI

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Composition of Species

Species traditionally are separated from one another primarily on a basis of morphologic characters. In dealing with phytopathogenic fungi, however, morphologically homogeneous complexes or aggregations of biotypes often have been divided into species which are distinguished by differential parasitism on different species or genera of host plants. These species are in turn frequently separated into cultural or parasitic races, strains or biotypes, often also on a basis of differential parasitism of varieties of host. In some phytopathogenic fungi it is important to distinguish between even smaller groups of biotypes, as in the distinction of Race 15 and Race 15B of *Puccinia graminis tritici* Erikss. & Henn. It is highly probable that most plant pathogenic fungi comprise large numbers of biotypes, whose most conspicuous and important differences are in physiologic characters and pathogenicity. When one or several of the biotypes behave with reasonable consistency they usually are designated as physiologic races. Reed (1, 2) recently has summarized pertinent information regarding such races, but the writers wish to emphasize the fact that many of these races may in turn comprise several or many distinct biotypes.

An analysis of the biotypes that comprise a species is a fundamental prerequisite to intelligent understanding of variability and variation. Stakman (3) emphasized the necessity of starting with the biotype, i.e., a population of genetically identical individuals, in attempting to distinguish between phenotypic variability, semipermanent modifications, genetic variation and changes resulting from differential behavior of biotypes in a mixed population. This concept is of utmost importance, not only in precise thinking about the problem, but also in analyzing the results and conclusions of experiments having to do with variation, and in exploring the underlying causes of variation.

Biotypes can be isolated and maintained with reasonable certainty in some plant pathogenic fungi. The sporidia of *Ustilago zeae* (Beckm.) Ung. normally are haploid, although occasional diploid sporidia may be produced, as had been proven by Christensen (4, 5), Eddins (6), and Chilton (7). These sporidia grow readily in culture and the asexually produced progeny of a single sporidium of *U. zeae* constitute a biotype, barring mutation. The same is true of other smut fungi which have a similar method of asexual reproduction. It is difficult, of course, to exclude the possibility of mutation, even in those monosporidial lines that appear to be absolutely constant, but the probability of maintaining a single biotype in these is far greater than is the case in many other fungi.

It has been shown by Stakman (8) and Stakman *et al.* (9) that *Ustilago zeae* comprises thousands of haploid biotypes that differ in cultural characters on artificial media, pathogenicity, sexual compatability, and morphologic characters. Stakman *et al.* (10) also showed that different chlamydospore collections may differ in pathogenicity, and Kernkamp & Petty (11) showed that the type of germination of the chlamydospores and the manner in which sporidia were produced varies greatly according to the cross or collection from which the chlamydospores were derived. The essential point is that if we are to gain a thorough understanding of the many complex types of variation which occur in this fungus and in other fungi, we must begin with a study of the biotypes which comprise the species.

Puccinia graminis Pers. also comprises seemingly innumerable biotypes. These can be grouped fairly satisfactorily into physiologic races, and the races into varieties that differ in pathogenicity, as shown by Eriksson (12), Stakman & Piemeisel (13), and Waterhouse (14) and that also differ in morphology, as shown by Levine (15). As determined by pathogenicity on a set of differential host plants, a physiologic race may comprise a single biotype or several apparently closely related biotypes.

Phenotypic Variability

It is difficult always to distinguish with certainty between phenotypic variability and genotypic variation, even in such fungi as *Ustilago zeae* and *Puccinia graminis tritici*, which have been thoroughly and critically studied in this respect. Stakman *et al.*

(9), Stakman, Tyler, & Hafstad (10), and Kernkamp (16, 17) showed that phenotypic variability may obscure genetic differences between monosporidial lines or biotypes of *U. zeae*, and Tyler (18) showed that this was true also of similar lines of *Sphacelotheca sorghi* (LK.) Cl., in which different biotypes appeared similar on several media but were quite distinct on another. Similarly Johnson (19), Johnson & Newton (20), Darley & Hart (21), Rodriguez (22), and Stakman, Levine & Loegering (23) have shown that the infection types produced by closely related biotypes or races of *P. graminis tritici* on varieties of wheat may be so greatly affected by light and temperature as sometimes to make recognition difficult. Gordon (24) has shown that this is true also of *P. graminis avenae* Erikss. & Henn., Peturson (25) of *P. coronata avenae* (Corda) Erikss. & Henn., Hassebrauk (26) of *P. triticina* Erikss., and Flor (27) of *Melampsora lini* (Schum.) Desm. This phenotypic variability on the living host is illustrated best by the rust fungi because of the distinctive infection types that they produce, but the same phenomenon occurs in other fungi also. In fact, the difficulty of distinguishing clearly between genotypic and phenotypic variation, a difficulty due sometimes to questionable genetic purity of the original material, and sometimes to a subsequent intermingling and confusion of the different types of variation, is one of the basic factors that has prevented precise analysis of the nature of variation in many fungi.

Semi-permanent Modifications

Caldis & Coons (28) studied the behavior of certain white variants that arose in monosporous cultures of *Septoria appii* (Br. & Cav.) Chester, *Sphaeropsis malorum* Peck, *Colletotrichum lindemuthianum* (Sacc. & Mag.) B. & C. and *Cladosporium fulvum* Cke. The variants of the first two species soon reverted to the parental types, but some of those from the latter two retained the variant characters for a long period, and then reverted more or less completely to the parental types. Caldis & Coons expressed the opinion that the variants were "semi-permanent variations which are different from the parent form somatically rather than genetically. These are the dauermodifications of Jollos . . ."

Stakman, Stevenson & Wilson (29) found that certain mutable and nonmutable monosporidial lines of *Ustilago zeae* increased their tolerance to sodium arsenite in the medium, so that lines

which originally tolerated only 2400 p.p.m. of this salt could, after ten transfer generations on the medium, tolerate 7000 p.p.m. A partially adapted line that at first produced colonies 15 mm. in diameter on a concentration of 3000 p.p.m., produced colonies 28 mm. in diameter after several transfers on media with the same concentration of sodium arsenite. The cultural characters of these lines were altered by their growth on media containing arsenic, but after several generations on arsenic-free media they gradually reverted to their original cultural types, and also lost the acquired tolerance to arsenic. Hirschhorn (136) studied the possible role of mutation in this adaptation, but concluded that the results could not be explained by observable mutations, although fairly numerous mutants were produced on arsenic media, some of the mutants tolerating more, and some less, arsenic than their parents. Gattani (30) studied the adaptation of monosporidial diploid lines of *U. zeae* to arsenic and malachite green, and obtained results essentially the same as those described above. These results are similar to those obtained by Jollos (31) with *Paramecium*.

Christensen (32), on the other hand, found that what appeared to be adaptation of monoconidial lines of *Gibberella zeae* (Schw.) Petch to malachite green, mercuric chloride and ethyl mercury phosphate was clearly due to the production of mutants. In the absence of mutation there was no perceptible increase in tolerance of the fungus to substances used as a result of successive transfers on media containing them. Some of the mutants were less tolerant to these substances than their parents, some about equally so, and others tolerated much more than the parents.

The above results show the necessity of caution in generalizing regarding the nature of variation in plant pathogenic fungi. The present evidence indicates that some monosporidial lines, or biotypes, of *Ustilago zeae* can gradually adapt themselves to arsenic and malachite green. While the genetic purity of the lines used might theoretically be questioned, the cultures originally were single biotypes, and on the basis of all available criteria they behaved as such during the entire course of the study. Christensen's results with *Gibberella zeae*, however, clearly were due to mutation. Much more investigation will be required in this field before we can state with finality the role which adaptation plays in the general field of variation in phytopathogenic fungi.

Mutation

Variants in monosporous lines of plant pathogenic fungi, evident as sectors, or patches, or in subcultures derived from single spores or hyphal tips, have been observed for a great many years. Although there was at one time considerable reluctance on the part of some investigators to call these variants mutations, it is now well established that mutation not only occurs in these fungi, but is very common in some of them. The question of the mutant nature of others of these variants is difficult to resolve because of lack of precise knowledge of the cytological condition of the initial material such as single spores or hyphal tips.

Although mutation has been reported in all classes of the fungi, there is a marked difference, not only among different genera and species, but also among biotypes within a species, in the frequency of mutation. Christensen (33) showed that some monosporous lines of *Helminthosporium sativum* P. K. & B. mutated freely in culture, while others remained constant over a period of years. Stakman *et al.* (9, 34) found that some haploid lines of *Ustilago zeae* produced hundreds of different mutants over a period of years, while other lines remained constant; they obtained good evidence that in this fungus the tendency to mutate was controlled by genetic factors.

Mutants may differ from their parents in almost any character, or combination of characters, by which a biotype may be recognized. Mutations in cultural characters have been reported more commonly than mutations in other characters, as would be expected, but mutants differing from their parents in sexual behavior, pathogenicity, and morphology, as well as in cultural characters, have been described. Some of these mutants differed from their parents only very slightly, and were detectable only by means of refined technics, but others were found that might well be considered different species from their parents, even when judged on the traditional morphological basis. Specific examples to illustrate the kind and range of mutations in phytopathogenic fungi will be discussed briefly.

Mutations in cultural characters.—Christensen (33), Christensen & Graham (35), Mitra (36), and Vallega (37) have described mutants of *Helminthosporium* species that differed from their parents in type of growth, color and topography of colonies, and

amount of sporulation. Brown (38), Eide (39), Leonian (40), Miller (41), Mitter (42), and Snyder & Hansen (43) have reported similar differences among mutants in *Fusarium* species, Schaal (44) in *Actinomyces scabies* (Thaxt.) Güss., and Walter (45) in *Ceratostomella ulmi* (Schwarz) Buisman. Allison (46), Christensen (47), Holton (48), Rodenhiser (49), and Stakman *et al.* (9) have described mutants in a number of smut fungi that differed strikingly from their parents in many cultural characters. Christensen (32) found mutants of *Giberella zeae* that were either more or less tolerant than their parents to various toxic materials, and Christensen & Davies (50) noted similar differences in *Helminthosporium sativum.*

Mutations in morphologic characters.—From a monosporous culture of *Pestalozzia funerea* Desm. which originally produced spores with three to five setae, Christensen (51) obtained a mutant whose spores had only two setae, and another mutant whose spores had one or occasionally no setae; the latter conformed more closely to the genus *Monochaetia* than to *Pestalozzia.* Brown (38), and Mitter (42) have reported mutants in *Fusarium* which differed considerably from their parents in certain morphologic characters. Miller (41) showed that the large macrospores which commonly appear in cultures of *Fusarium* grown on artificial media are mutants, and he also presented good evidence that the sporodochia produced by isolates of *Fusarium* growing on agar media are in reality patch mutants. LeClerg (52) described mutants of *Rhizoctonia solani* Kühn which differed from their parents in diameter of hyphae.

Mutations in color.—White or albino mutants are very common in a great many fungi grown on artificial media. Often, but not always, this is associated with loss of sporulation. Greany & Machacek (53) found a fertile white mutant in *Helminthosporium sativum* that had been exposed to ultraviolet radiation. One of the writers has found a fertile albino mutant to appear commonly in old cultures of *Hormodendrum resinae* Lindau. Brierley (54) described a variant of *Botrytis cinerea* Pers. with colorless sclerotia. Many of the mutations reported in *U. zeae* by Stakman *et al.* (9, 34, 55) involved color as well as other characters, and many of the mutations in cultural characters summarized above also have included changes in color. Mutations in which the color differed from the normal has been reported by Newton & Johnson (56),

Stakman, Levine, & Cotter (57), and Waterhouse (14) in *Puccinia graminis tritici;* by Cotter & Levine (58) in *P. gr. secalis* Erikss. & Henn., by Gordon (59) in *P. gr. avenae,* by Johnston (60) in *P. rubigo vera tritici* (Erikss.) Carl., and by D'Oliveira (61) in *P. anomala* Rostr. The buff smuts described by Holton (48, 62) and by Moore & Allison (63) were mutants in which the color had been lost from the spore wall.

Mutations in pathogenicity.—Loss of virulence of phytopathogenic fungi when cultured on artificial media is a relatively common phenomenon, and frequently is associated with mutation. Christensen (33), Christensen & Davies (50), and Mitra (36) found a large number of mutants in *Helminthosporium* species which differed from their parents in pathogenicity. Most of these were less pathogenic than the parents, but some were decidedly more pathogenic. Evidence presented by Brown (38), Mitter (42), and more recently by Miller (41) indicates that loss of virulence of isolates of *Fusarium* grown on artificial media is due to the occurrence of mutants which not only are less pathogenic than the parents but which also are able to overgrow the parents in culture. Loss of virulence was common among the mutants reported by Stakman *et al.* (9) in *Ustilago zeae,* and by Shay & Keitt (64) in *Venturia inaequalis* (Cke.) Wint. Stakman, Levine & Cotter (57) and Newton & Johnson (65) have recorded several mutations in pathogenicity in *Puccinia graminis;* Roberts (66) reported one in *P. rubigo vera tritici;* and Gassner & Straib (67) found several in *P. glumarum* (Schm.) Erikss. & Henn. Most of these were less virulent than their parents on the varieties on which they were tested. Because the expression of virulence is influenced by a great variety of factors in the environment, as well as by the genetic factors of the fungus itself, and so is not usually subject to extremely precise measurement, it is probable that many minor mutations of this kind go unnoticed. Also a mutation which occurred in only one nucleus in the dicaryotic stage of the rust fungi might be recessive and therefore not apparent until it was present in the homozygous condition. Similarly if a heterocaryotic condition prevailed in the mycelium or spores of a fungus, mutation might be hidden until a chance assortment of nuclei allowed it to be expressed.

Influence of specific environmental factors on the production of mutants.—Barnes (68), Christensen (69), and Mitra (36) found that if cultures were exposed for a time to high temperature, the

rate of mutation was increased. Brown (38), Christensen (33), Stakman *et al.* (9) and Tyler (18) showed that the chemical composition of the medium on which the fungus was grown had a great influence on the rate of mutation. Dimock (70) concluded that variation in *Fusarium* species was induced by high concentration of zinc salts. Christensen & Davies (50) found that a given stimulus, such as staling products, would induce one biotype of *Helminthosporium sativum* to mutate, but had no effect on another biotype. Miller (41) found that certain isolates of *Fusarium*, although extremely variable on agar media, remained constant for years when cultured in soil. Orton (71), however, had conclusive evidence that "several strains of *Fusarium niveum* Smith may dissociate as freely in the soil as upon laboratory media." There is abundant evidence to suggest that many phytopathogenic fungi are relatively stable in nature, as compared with their behavior when grown on artificial media. This is of some significance to the practical plant pathologist, since it often is desirable to maintain biotypes of constant and known virulence for testing disease resistance of economic plants.

Heterocaryosis

Heterocaryosis, the presence of two or more genetically different nuclei in the same hyphal cell or spore, first described by Hansen & Smith (72), apparently is a rather common phenomenon in some fungi. Dickinson (73), Hansen (74), and Hansen & Smith (72) have shown that when different biotypes of a species are growing together so that their hyphae are intermingled, the hyphae of one biotype will fuse with those of the other, and that a reassortment of genetically different nuclei may give rise to variants. Beadle & Coonradt (75) and Dodge (76) have shown that heterocaryotic races of *Neurospora* species which arose from hyphal fusion of two different monocaryotic lines having the same factors for sex, differed greatly from their component lines in physiologic behavior. Hansen (74) and Hansen & Smith (72) as well as some other investigators have suggested that heterocaryosis is one of the chief methods by which new races of Fungi Imperfecti arise.

The relative importance of heterocaryosis versus mutation, in the production of new biotypes of plant pathogenic fungi, remains at present largely in the field of speculation. Christensen & Davies

(50) found that although hyphal fusions occurred between different biotypes of *Helminthosporium* species, and thus a heterocaryotic condition presumably arose in the mycelium, the spores formed on this mycelium were originally uninucleate, so that the heterocaryotic condition was not perpetuated in the spores. The cells of these spores later became multinucleate, and the cells of the mycelium derived from them also were multinucleate, but all of these nuclei were descended from the one original nucleus present in the young spore, so that the occurrence of several nuclei in the same cell of a spore or hypha is not in itself evidence of heterocaryosis. Even in those specific cases where heterocaryosis has been proven to be a source of variation, there is no reason to suppose that the nuclei involved in the association do not occasionally mutate, and thus compound the variation. Indeed, it seems reasonable to suppose that mutation might frequently be responsible for the origin of a heterocaryotic condition in those fungi which have more than one nucleus per cell.

It is important to realize that mutation has been observed in many haploid lines of different species of smuts, where heterocaryosis does not occur, and mutation has also been observed in other fungi in which the hyphal cells and spores are predominantly or exclusively uninucleate, so that heterocaryosis would be largely eliminated from consideration, as in *Colletotrichum distructivum* O'Gara as reported by Chilton (77), in *Cladosporium fulvum* as reported by Langford (78), in *Venturia inaequalis*, as reported by Keitt & Langford (79), and in *Ceratostamella ulmi* as reported by Walter (45).

Hybridization

Hybridization is one of the principal causes of genetic variation in those phytopathogenic fungi which reproduce sexually. There is sometimes extensive recombination between biotypes and varieties within a species, between well defined species, and even between perfectly valid genera. As far as the writers are aware, intraspecific combinations of plant pathogenic fungi have never produced hybrids that could be designated as new species, but certain interspecific and intergeneric crosses have produced hybrids that could be considered as new species.

Hybridization between biotypes within a species.—Stakman & Christensen (80) in 1927 crossed many different races of *Ustilago*

zeae, and stated that in this fungus there were multiple factors for sex and pathogenicity. Tyler (18) made ten crosses between the sporidia isolated from three germinating chlamydospores from a single smut sorus of *Sphacelotheca sorghi*. The progeny of these crosses differed among themselves in size of chlamydospores, size of sori produced on the host, hardness of sori, length of time required for the spores to germinate, and in the degree of stunting caused on the host. Vaheeduddin (81), continuing work with these same isolates, found that the character of brown peridium bred true through six generations. Sporidia isolated from spores of sori with brown peridium were mated with those from spores of a sorus with gray peridium. The progeny segregated into groups with brown, grayish brown, and gray peridium. From the progeny of one of these crosses Vaheeduddin isolated a new pathogenic race of sorghum smut. He concluded that the species comprised a very large number of freely interbreeding biotypes.

Keitt *et al.* (79, 82) mated single ascospore cultures of *Venturia inaequalis* whose characters, both pathogenic and cultural, were well known and constant. Ascospores from these hybrids yielded cultures comprising the parental types, or combinations of them. The factors for pathogenicity segregated in the first or second division in the ascus; the infection type produced by each biotype on a given host apparently depended on genes in a single locus, with multiple alleles determining infection type on different varieties of the host.

In similar work with *Venturia pirina* Aderh., Langford & Keitt (83) found that hybrids between single ascospore cultures differed in length of time required to produce mature perithecia, and in number of ascospores produced. The single ascospore lines isolated from these hybrids differed in pathogenicity on certain varieties of pears, and this was found to be an inherent character. Their work provides strong evidence that, in both of these fungi, genetic combinations between lines and subsequent segregation is responsible for much of the variation encountered in nature. Chilton *et al.* (84) likewise present evidence from experimental hybridization between different strains of *Glomerella* which suggests that such hybridization may be a fertile source of variation in this fungus in nature.

Hybridization between varieties.—Stakman *et al.* (85) suggested in 1918 that *Puccinia graminis* might be hybridizing on barberries,

with the consequent production of new races, but the discovery by Craigie (86) in 1927 of the sexual nature of the pycnia of this fungus provided the basis for experimental exploration of this possibility. Subsequent to Craigie's discovery, numerous crosses were made between different races and varieties of *P. graminis* by Waterhouse (87), Johnson, Newton & Brown (88), Newton, Johnson & Brown (89, 90), Stakman, Levine & Cotter (57), Levine, Cotter & Stakman (91), and Cotter & Levine (137). Much of this work has been summarized by Craigie (92) and by Johnson & Newton (93), and only sufficient material will be presented here to illustrate the essential principles involved.

From two individual crosses between race 36 of *Puccinia graminis tritici* and *P. gr. agrostidis* Erikss. & Henn., which are sufficiently distinct morphologically to be designated as varieties of the species, Stakman, Levine & Cotter (57) obtained twelve different races of *P. gr. tritici*. Three of these races had never been found in nature, one was known only from Portugal, and one had been isolated in nature only from aecia. Eight of these races were obtained from a single aecium. From the progeny of reciprocal crosses between race 36 of *P. gr. tritici* and race 11 of *P. gr. secalis* they isolated races 9 and 11 of *P. gr. secalis*, and races 15, 21, 32, 36, 57, 67, 70, and 71 of *P. gr. tritici*. The race 67 of *P. gr. tritici* isolated from this cross was, on the differential hosts tested, identical with the race 67 isolated from the previously mentioned cross between race 36 or of *P. gr. tritici* and *P. gr. agrostidis*.

Some of the races resulting from such crosses were more pathogenic on certain host varieties than either of the parents. A number of biotypes of several of the races also were found in this hybrid material, deviating in spore color and other characters from the average of the races to which, on a basis of pathogenicity, they obviously belonged.

Cotter & Levine (137) made 492 intervarietal matings between *Puccinia graminis agrostidis* and *P. gr. poae* Erikss. & Henn., *P. gr. secalis*, *P. gr. avenae*, and *P. gr. tritici*. Thirty-six per cent of these matings resulted in aecia. From the progeny of crosses between *P. gr. tritici* and *P. gr. agrostidis* they isolated twenty-two races of *tritici*, two of *hordei*, and one of *varicola*, the latter a new variety within this composite species. From the progeny of crosses between *secalis* and *agrostidis* they isolated thirteen races of *tritici*,

five of *secalis*, one of *avenae*, one of *hordei;* and from the progeny of crosses between *poae* and *agrostidis* four races of *tritici* were isolated. They state that

"Most of the races obtained are well known and many are of common occurrence and wide distribution. But eight of the *tritici*, four of the *hordei*, and the one *varicola* races have thus far not been found in nature, either on alternate or gramineous hosts."

Johnson, Newton & Brown (88) and Newton, Johnson & Brown (89) selfed several of the races of *Puccinia graminis tritici* and found that most of them were heterozygous for pathogenicity; when race 30 was selfed, three races were isolated from the progeny, and when race 95 was selfed, nine races were isolated from the progeny, and when race 53 was selfed, eighteen races were isolated from the progeny.

Flor (94) crossed race 22 of *Melampsora lini*, from South America, with race 24, from the United States. In the F_2 progeny he identified sixty-four different races, of which sixty-two had not been found in nature or previously described. Some of these races were more virulent than either of the parents on certain of the host varieties.

It should perhaps be emphasized that the amount of labor involved in identifying different races of these rust fungi on a series of differential hosts precludes the possibility of exploring the total variation that might occur among the progeny of even a single cross. The results already obtained, however, have established the general principle that these rusts, and probably many others also, regularly hybridize in nature; that some of the common and widespread races of *Puccinia graminis tritici* are actually heterozygous for pathogenicity and almost certainly are the products of hybridization in nature; that from a single cross a relatively large number of pathogenically distinct races or even varieties may be produced, which may differ from the parents in virulence on a given host and also in host range; and that in all probability many of the races encountered in nature have arisen through hybridization between genetically diverse parents.

Hybridization between species.—Holton (48, 62) and Hanna & Popp (95) crossed *Ustilago avenae* (Pers.) Jens., which has echinulate spores and produces a loose sorus, with *U. levis* (Kell. & Sw.) Mag., which has smooth spores and produces a compact sorus. The F_1 progeny had echinulate spores and a loose sorus like that of

the *U. avenae* parent, so their hybrid nature could not be determined by microscopic inspection. From the progeny of one of the crosses between these two species Holton isolated a smut with buff colored spores, somewhat similar to a buff smut of wheat which had been described from nature by Campagna (95) and Murphy (97). Later Holton (62), working with the same two species of oats smut, found a hybrid between them which was considerably more virulent on certain oat varieties than either of the parents. On the basis of this and considerable additional evidence gained from testing field collections of these smuts, Holton concluded that ". . . hybridization between species plays an important role in the development of new pathogenic strains of the oat smut fungi."

Allison (46) found that the F_1 progeny of a cross between *Ustilago hordei* (Pers.) Kell. & Sw., and *U. medians* Bieden. had spores with echinulate walls, like those of the *U. medians* parent. Flor (98) showed that *Tilletia tritici* (Bjerk.) Wint. would hybridize readily with *T. levis* Kühn; and Rodenhiser & Holton (99), who tested twenty-four collections of *T. tritici*, twenty-nine of *T. levis*, and a number of miscellaneous collections not specifically identified, on ten varieties of winter and spring wheats over a three-year period at three different stations, considered that some of these collections were hybrids between *T. tritici* and *T. levis*. Christensen & Rodenhiser (100) emphasized the importance of taking into account this hybridization in the development of smut-resistant varieties.

Hybridization between genera.—Vaheeduddin (81) obtained hybrids between *Sphacelotheca sorghi* and *Sorosporium reilianum* (Kühn) McAlp. Some of these hybrids resembled one parent in size and shape of sorus and morphology or spores, some produced sori resembling those of *Tolyposporium filiferum* Busse, and some were quite distinct from any of these three genera. A notable feature of these results was the evidence of pronounced hybrid vigor in some of the intergeneric hybrids.

Zundel (101) in 1939 stated that certain collections of *Sphacelotheca panicimiliacei* (Pers.) Bubak on *Panicum miliaceum* closely resembled *Sorosporium syntherismae* (PK) Farl. Martin (102) mated sporidia isolated from germinating chlamydospores of these two fungi. Twenty-three of forty-seven intergeneric matings produced mature chlamydospores on *Panicum miliaceum*, a host for both of these smuts, and four of fifteen produced mature chlamy-

dospores on *Panicum capillare*, supposedly a host of *S. syntherismae* only. Different hybrid combinations differed considerably in pathogenicity on various hosts. The sori produced by the hybrids resembled those of one parent in shape and size, but were intermediate between the two parents in the aggregation of spores into clumps within the sorus, while the wall markings of the spores resembled those of the other parent. Size of the spores of these intergeneric hybrids varied independently of the wall markings. The most significant result, however, is the evidence of extension of host range resulting from hybridization.

Evidence of Variation in Nature

Although there is abundant evidence that very numerous biotypes of most plant pathogenic fungi occur in nature, the evidence as to their mode of origin must remain largely circumstantial. Christensen & Graham (35) isolated numerous parasitic races of *Helminthosporium gramineum* (Rab.) Erik. from barley, and obtained as many as ten distinct cultural types from a single barley plant. Borlaug (103) isolated a large number of cultural and pathogenic biotypes of *Fusarium lini* Boll. from flax, and had circumstantial evidence to indicate that different pathogenic biotypes may prevail in the soil of different wilt nurseries. Voorhees (104) found that several biotypes of *Physalospora rhodina* (B. & C.) Cke. could be isolated from a single perithecium, and even from a single ascus; and Keitt & Langford (79) found the same to be true of *Venturia inaequalis*. Allison (46), Becker (105), Hanna (106), Martin (102), Rodenhiser (49), Nicolaisen (107) and others have isolated many different biotypes of smut from a single sorus, and it is not uncommon to isolate four genetically different haploid biotypes from a single germinating chlamydospore of some smut fungi.

In *Puccinia graminis* there is convincing evidence as to the way in which new parasitic races arise. By selfing several races of this fungus, Newton, Johnson & Brown (89) showed that the majority of them were heterozygous for pathogenicity; from aeciospores produced as a result of self fertilizing race 53 they isolated eighteen races. Stakman *et al.* (108) found that in samples of rust collected near barberry bushes, about one of every four tested was a different race, while in those collected at random in areas where no barberry bushes were present, one in about every sixty was a new race.

Practical Importance of Variation in Phytopathogenic Fungi

In taxonomy.—Evidence presented in the preceding sections indicates the range and kind of variation in some of the common plant pathogenic fungi. Each of those species studied most extensively has been found to comprise large numbers of biotypes, differing from one another in physiological or morphological characters, or both. New biotypes continually are being produced by mutation, hybridization, or by other means, and so these species are not constant and static, but shifting and fluctuating. Of what significance is this from a taxonomic viewpoint?

The worth of any taxonomic system depends to a considerable extent upon the validity of criteria chosen to separate groups. Useful and usable systems must take into account the range of phenotypic variability and the genetic variation in the organisms concerned, if distinctions without differences are to be avoided. A knowledge of such variation is basic to a sound taxonomy. The following examples serve to illustrate this.

Leonian (109, 110) and Tucker (111), in extensive studies of numerous species in the genus *Phytophthora*, found that almost all of the morphologic and physiologic characters used to separate species varied greatly. This variation often was even more extensive when the fungi were growing on their natural hosts than when growing on artificial media in the laboratory. Mehrlich (112), found variations within the species *P. cinnamomi* Rands, *P. palmivora* (Butl.) Butl. and *P. parasitica* Dastar "similar to the physiologic strains of rust fungi." Because of this he questioned the validity of separating species of *Phytophthora* on the basis of differential parasitism on a single host, and suggested that *P. cinnamomi* and *P. cambivora* (Petri) Buis. be combined into one species. He also found that ability to grow on corn meal agar at 35° C. was not a valid distinguishing character of *P. parasitica*, since some strains of this species from Hawaii would not grow at this temperature.

Blackwell *et al.* (113) found that the genus *Pythiomorpha*, erected shortly after 1900 to include what appeared to be an unusual water mold, and in which several species had been since described, was only *Phytophthora* growing in water. Thus, when *Pythiomorpha gonapodyides* Peterson growing on an apple in a pond was cultured on agar, it was found to be identical with *Phytophthora megasperma* Drechs. Similarly Mehrlich (112) found *Pseu-*

dopythium phytophthoron Sideris to be only a strain of *Phytophthora cinnamomi*. These cases illustrate the pitfalls into which a taxonomist may easily blunder in describing new organisms from "natural" media if the range of phenotypic variability is not investigated thoroughly.

The genus *Fusarium* has long presented seemingly insoluble difficulties to the taxonomist, difficulties which arise in part from the tremendous range of variability of many species when grown in culture. The monumental works by Appel & Wollenweber (114), and Wollenweber *et al.* (115) resulted in a separation of the genus into 65 species, 56 varieties, and 22 forms. While this reduced what had been essentially taxonomic chaos in this genus to a semblance of order, it was by no means perfect order. Brown (38), Snyder & Hansen (116) and others found different single spore isolates from one culture of *Fusarium* to vary so much that they would have to be placed not only in different species but even in different sections of the genus. Snyder & Hansen (116) reduced the number of species to eight, separated on the basis of morphological characters which took into account the great degree of variation encountered. Miller (41) found that most of the strains of *Fusarium* which he investigated remained constant when grown in soil. Grown on agar, however, the original wild types soon were overgrown by mutant types. The sporodochia formed in cultures of *Fusarium* he found to be patch mutants, characterized by the production of more numerous and larger macroconidia. His contention that "If these structures are the result of mutations occurring in the cultures studied, then it is reasonable to question whether such measurements can be of reliable diagnostic value," seems to be justified, at least in the cultures he studied.

Christensen & Graham (35) found striking differences in size, shape, and number of septations of spores of different races of *Helminthosporium gramineum*. One race produced spores which averaged 65.7 microns in length while the spores of another race averaged 101.4 microns. Some of these races differed considerably from the generally accepted description of the species, which had been considered by most mycologists familiar with it to be rather stable.

The common smuts of cereal plants are generally held to be a relatively stable group taxonomically. Yet extensive work, cited in the previous sections, has shown that there is scarcely a single character used to separate species and genera which does not vary

appreciably. Often this variation transcends species limits. Interspecific and even intergeneric hybrids have been produced in the laboratory, and evidence cited above indicates that the same phenomenon occurs occasionally and sometimes frequently in nature. The hybrid nature of some chlamydospores can not be recognized by microscopic examination. Tervet (117) describes a case in which one collection of oat smut, originally identified as *Ustilago avenae*, came out as apparently pure *U. levis* after several passages through Black Mesdag oats. As he points out, the original collection may have been a mixture of the two species, or a hybrid between them, or may have included both. This suggests the difficulty of categoric separation of closely related species. In view of this variability, the recent reduction to synonymy of a number of closely related forms and species of the common smuts by Fischer (118) seems a commendable advance in the taxonomy of this group. It is unlikely that the range of variation in the common smuts of cultivated plants is unique; similar variation can be expected in those species which have not yet been studied thoroughly, and, should this be the case, it may eventually involve laborious revision of those species described, often from single collections, without taking this into account.

Similar evidence of variability and consequent confusion could be cited for such common genera of plant pathogenic fungi as *Aphanomyces*, *Botrytis*, *Colletotrichum*, *Cytospora*, *Gloesporium*, *Ophiobolus*, *Phoma*, *Pythium*, *Sclerotinia*, and a host of others. One of the obvious reasons why many of the groups of fungi are in a relatively muddled taxonomic state is that we do not know the total range of variation within and between groups, and so are uncertain what characters are the more valid ones to use in separating natural or even artificial groups. An experimental approach to these problems, embodying the concept of variation and the technics now available for studying it, ought to become an integral part of modern taxonomy of pathogenic fungi.

In development of resistant varieties.—More than twenty years ago Stakman (119) emphasized the necessity of knowing the number, pathogenic capabilities and distribution of physiologic races of pathogenic fungi, in order to produce and maintain varieties of crop plants resistant to them. In the summary of a ten-year survey of races of *Puccinia graminis tritici* by Stakman *et al.* (120) the authors state,

"The population shifts of physiologic races show the practical need for extensive replication in time and space in testing varieties for stem rust resistance, unless the varieties are exposed to artificially induced epidemics created by inoculating with all races that occur or are likely to occur in the region for which the variety is intended."

The case of Ceres wheat, summarized by Hart (121) illustrates this point. This rust-resistant variety was distributed in 1926, and by 1934 was grown on more than four million acres. Race 56 of *P. graminis tritici* was first identified in 1928; in 1930 it comprised only 0.2 per cent of the wheat stem-rust population, as determined by Stakman *et al.* (120), increased to 1.0 per cent of the population in 1931, to 2.1 per cent in 1932, 3.7 per cent in 1933, then increased rapidly to make up 33 per cent of the population in 1934 and 66 per cent in 1938. Ceres was very suseptible to this race, and in the severe stem rust epidemics of 1935 and 1937, in which race 56 predominated, Ceres was so heavily damaged that it ceased to be generally cultivated.

Murphy & Levine (122) reported that the oat variety Victoria, resistant to thirty-seven physiologic races of crown rust, and widely used as a parent in oat breeding, was susceptible to race 41, which was collected only once, in Texas, and which had hitherto not been identified.

The oat varieties Vicland and Tama, released shortly after 1940 and widely grown in the chief oats-producing states in the Midwest, were resistant to the races of *Puccinia graminis avenae* that were prevalent when these varieties were released. They were, however, susceptible to race 8, which at that time had a very limited distribution. Stakman & Loegering (123) pointed out in 1944 that race 8 of *P. graminis avenae* was increasing in 1943, and might constitute a real danger to these oat varieties, and to others of similar parentage. This danger became a reality in 1945 and 1946, when race 8 increased rapidly and caused appreciable loss. Moore *et al.* (124) stress the need to obtain oat varieties resistant to all prevalent races of this fungus.

Durum wheats were long considered to be more resistant to bunt than the common wheats in the United States, although Reichert (125) stated that in Palestine the reverse was true. Between 1925 and 1930, bunt became increasingly prevalent and destructive on the durum wheats grown in the Midwest and Holton (126) found this increase to be due to a race of *Tilletia tritici* previ-

ously unknown. A similar situation prevailed in the common wheats. In 1922 Stephens & Woolman (127) listed twenty varieties of wheat resistant to bunt when tested in Oregon, and Schafer *et al.* (128) in 1926 reported four varieties of wheat very resistant to bunt in Washington. Rodenhister & Holton (99, 129) later found all of these supposedly resistant varieties to be susceptible to one or more races of *T. levis* or *T. tritici*. Christensen & Rodenhiser (100) state, "It has been pointed out repeatedly that no other factor has contributed more to the difficulty of obtaining smut resistant varieties of cereals than physiologic specialization in the pathogens."

Similar phenomena have been encountered in many other plant pathogens, and it seems likely that the same general principles can be applied to their solution. In Europe, varieties of potatoes bred for resistance to late blight, caused by *Phytophthora infestans* (Mont.) DeB., were subsequently found by Müller (130) to be heavily infected by previously unknown races of the fungus. Borlaug (103) found circumstantial but convincing evidence that different races of *Fusarium lini* predominated in different wilt nurseries in the United States and Canada. Of nine advanced lines of flax grown in the wilt nursery at University Farm, St. Paul, Minnesota, seven had little wilt. When the same lines were grown in the wilt nursery at Ottawa, Canada, however, wilt ranged from 20 to 68 per cent in all nine. He stressed the need for occasionally adding new races of *Fusarium lini* to the soil in wilt nurseries, and the desirability of testing promising new lines of flax in wilt nurseries in different regions. Christensen & Graham (35) pointed out the value of using numerous races of the fungus in developing varieties of barley resistant to *Helminthosporium gramineum*, and Schaal (44) recommended that in breeding for scab resistance, potato selections be tested in uniform scab test plots in various potato growing areas, where different races of *Actinomyces scabies*, as well as different environmental conditions, would prevail.

At the present time, exposing improved lines of plants which are being bred for disease resistance, to artificially created epidemics in disease gardens, where they are inoculated with numerous collections of each organism concerned, or of testing them at a number of stations throughout the region where they are to be grown, has become almost standard practice. Recent experience with stem rust of oats, leaf rust of wheat, dwarf bunt of wheat,

Helminthosporium blight of oats and other diseases warn us that tests must be made even more extensive and more thorough than has so far been possible if agricultural production is to be stabilized.

In quarantines.—Information on the relation of races and varieties of phytopathogenic fungi to plant quarantines has been summarized by McCubbin (131), Hunt (132), and Stakman (133), and only a few of the more outstanding examples will be cited here. Many races of *Puccinia graminis tritici* present in the United States are not found in Australia, Argentina, India, China, or the other principal wheat growing areas of the world where stem rust is an economically important disease. Garcia-Rada *et al.* (134) in 1942 reported the existence of a race of stem rust in Peru, identified as race 189, capable of causing heavy infection on a large number of different varieties of wheat, including most of those now grown in the chief wheat producing areas of the United States. Flor (94) showed that races of flax rust present in Argentina were virulent on some of our flax varieties resistant to indigenous races, and that some of the races of this fungus present in the United States were capable of attacking varieties of flax now considered resistant to rust in Argentina. Borlaug (103) found that some of the races of *Fusarium lini* isolated from flax from Argentina were capable of producing severe wilt in all varieties of flax included in his tests. He was able to isolate *F. lini* from seeds and plant parts collected from commercial shipments from Argentina, and the fungus remained viable in this material for at least a year after harvest. The fact that some of the flax imported from South America is planted in fields, and that some may be spilled or scattered on the ground around terminals or along rights of way make the possibility of such introduction a very real one.

Evidence by a number of workers indicates that races of the principal cereal smuts are not at all uniformly distributed among the countries in which they are economically important. That a similar situation has not yet been found to exist in many other important plant pathogens may be due to the fact that they have not been investigated so thoroughly from this standpoint as those here cited.

In epidemiology.—The extensive surveys of races of *Puccinia graminis tritici* by Stakman *et al.* (120, 135) prove that different races of this fungus prevail in southern Mexico than in northern Mexico and the United States; and that in the United States the

different races fluctuate in prevalence as the host varieties change and as the environmental conditions alter from year to year. The striking increase in bunt in durum wheat in midwestern United States between 1925 and 1930, found by Holton (126) to be due to a race of *Tilletia tritici* new to the region already has been mentioned. Work already cited by Flor (94) with *Melampsora lini*, Borlaug (103) with *Fusarium lini*, and Christensen (33) with *Helminthosporium sativum* provides additional evidence that we must explore as completely as possible the modes of origin, pathogenic capabilities, and reaction to environmental factors of the different races and biotypes of the organisms involved if we wish to analyse the forces that contribute to the making of plant disease epidemics.

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VARIATION IN PHYTOPATHOGENIC VIRUSES

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Introduction

When phytopathogenic viruses multiply they apparently produce exact replicas of themselves. Hence, they conform to the first law of heredity which is that "like breeds like." But they also conform to another law of heredity which similarly stated is that "like breeds unlike." These paradoxical statements may be harmonized by explaining that plant viruses usually multiply without change but that occasionally, rarely in some instances, more often in others, they produce variants which are more or less unlike the entities from which they spring. In this respect they behave like bacteria, fungi, and other organisms.

Recognition and study of plant virus variants was promoted by three discoveries that greatly facilitated experimental efforts to understand virus relationships (1). The first of these was a demonstration that a seemingly unlimited number of yellow and necrotic type viruses could be isolated from small, irregular-shaped, bright yellow spots that occasionally appeared on leaves of tobacco plants inoculated with ordinary tobacco mosaic virus. McKinney (2) was the first to observe and study these spots. For some time he suspected that they might be due to contaminations. However, when he found that different samples of ordinary tobacco mosaic virus obtained from various sources and apparently pure in the sense that they were free of contaminations, gave yellow spots in about the same numbers as the original sample, he modified this view. Each of seventeen tobacco mosaic virus samples obtained from seven States of the United States and from Hawaii, England, and the Canary Islands gave yellow spots after inoculation in tobacco plants (3). This general association of yellow mosaic with the ordinary green tobacco mosaic from different sources led McKinney to suggest that "viruses may become altered locally in the plant, thus producing mutations.' In 1933 Jensen (4) reported that he had been able to isolate a whole series of yellow mosaic type viruses from bright yellow spots that regularly appeared in leaves of tomato and tobacco plants affected by tobacco mosaic. It was concluded that they were variant strains and that they arose from

tobacco mosaic virus. In further work with yellow spots on plants having tobacco mosaic Kunkel (5) isolated 130 different variants and passed them in series in tobacco plants. In 1934 Price (6) reported isolation of several different strains of yellow cucumber mosaic virus from bright yellow spots that occasionally appeared on leaves infected with ordinary cucumber mosaic virus. Proof that yellow and necrotic type strains of tobacco and cucumber mosaic viruses regularly arise in plants affected by ordinary tobacco and cucumber mosaic stimulated work on virus strains.

In 1928 Purdy (7) reported that tobacco mosaic virus behaved as an antigen when injected into rabbits. Subsequent work confirmed this report and proved that closely related viruses have similar antigenic properties (8 to 13). Serological reactions thus became a tool for use in determining whether or not two different viruses were closely related.

In 1931 Thung (14) described experiments in which a yellow mosaic virus, isolated from plants having tobacco mosaic, was used. When tobacco plants affected by this yellow mosaic were inoculated with tobacco mosaic virus they failed to develop symptoms of tobacco mosaic, and when plants having tobacco mosaic were inoculated with the yellow mosaic type virus they failed to develop symptoms of yellow mosaic. Thung thus proved that plants with the yellow mosaic became immune to tobacco mosaic and vice versa. In 1934 Kunkel (15) showed that leaves of *Nicotiana sylvestris* plants could be immunized against infection by the virus of aucuba mosaic of tomato through a previous inoculation with tobacco mosaic virus or with attenuated strains of this virus. The immunity acquired seemed to be specific for it was found that infection of leaves by either cucumber mosaic virus or tobacco ringspot virus afforded no protection. Thus another kind of immunological reaction became available for testing plant virus relationships. Both methods have proved valuable in work with viruses that are transmissible mechanically by means of juices. Both have been widely used during the past fifteen years.

Survey of Virus Mutants

Tobacco mosaic strains.—The mutants of tobacco mosaic virus have received more attention than have those of any other plant virus disease. This has resulted partly from the fact that tobacco mosaic virus is widely known and partly from the fact that its mutants are numerous, easy to isolate and easy to transmit. Up

to the present time more than 400 strains of tobacco mosaic virus have been isolated or reported as occurring in nature. When it is remembered that thousands of bright yellow spots, from which yellow mosaic type strains of tobacco mosaic virus could be isolated, appear in every tobacco field where tobacco mosaic is generally prevalent, it is clear that only a small beginning has been made in becoming acquainted with even these best known types of variants. Present concepts regarding the nature of tobacco mosaic virus mutants are derived from the relatively few that are known and, hence, may be somewhat incorrect. On the other hand, it is possible that the strains already studied represent a fair sample of those that are produced and that further isolations may not yield any new factors of variability. Before discussing the ways in which variant strains of tobacco mosaic virus differ from the ordinary or parent strain, it may be advantageous to characterize the latter briefly.

Ordinary tobacco mosaic virus consists of submicroscopic, rod-shaped particles about fifteen millimicrons in breadth and of varying lengths, composed chiefly of nucleoprotein and possessing a high degree of resistance to heat, desiccation and deleterious chemicals. In tobacco plants of many different varieties it multiplies rapidly and causes a green distorting type mosaic disease of moderate severity. It has a wide host range amongst dicotyledonous plants in which a correlation seems to exist between accepted taxonomic classification and susceptibility. Its time, place, mode of origin, and relationships to other plant parasites are unknown.

Under ordinary conditions the parent strain of tobacco mosaic virus appears to multiply faster and to move more rapidly in tobacco than other strains (16). Hence the parent strain overgrows others that may be present or may arise from time to time. It probably is because of this that tobacco mosaic virus causes such a uniform type of disease throughout the world. However, in spite of its great uniformity there appear to be fluctuations in the disease-producing propensities of the so-called parent strain. Johnson & Valleau (17) state that it is not unusual to find different strains of tobacco mosaic virus predominating on each farm in old tobacco-growing areas. Johnson (18) has shown that mild strains get the upper hand when tobacco mosaic virus is passed in sea holly. Nolla (19) obtained a mild form of tobacco mosaic virus from tobacco plants of the resistant variety Ambalema inoculated with ordinary tobacco mosaic virus. Valleau (20) also obtained

mild strains of tobacco mosaic virus by passing the ordinary strain in Ambalema. Apparently mild strains multiply faster than the parent strain in both Ambalema and sea holly.

The most striking way in which tobacco mosaic virus mutants differ from the parent strain is in severity of symptoms produced. Many dwarf (21) and malform tobacco leaves; a few cause distortions of the enation type on tobacco (22) and tomato (23, 24); several cause more or less filiforming in leaves of both tobacco (25) and tomato. The parent strain never causes striping or spotting of stems of tobacco or tomato but many of the yellow strains do and some cause spotting and striping of tomato fruits (26). Many strains produce different shades of yellow and white mosaic type patterns (4, 27, 28, 29). A few cause various shades of light green and yellow in ringspot patterns (30, 31, 32). Many strains of both green and yellow mosaic type viruses cause more or less necrosis (30, 33, 34). Thus it is seen that the different varieties of tobacco mosaic virus cause a whole series of symptom complexes, varying from those in which signs of disease are almost or entirely absent to those in which necrosis is a prominent feature. All have similar chemical and physical properties.

Mutants isolated directly from the parent strain give strong precipitin reactions. Twenty-five different white, yellow, necrotic, symptomless and green mottling type strains isolated or assembled by Jensen were found to show such close serological relationships that Chester (35) considered them strains of one virus. Holmes' masked strain of tobacco mosaic virus was found to be closely related to the parent strain serologically (24). Serological studies indicate that certain tobacco mosaic virus strains present in nature are not so closely related to the parent strain as are those isolated directly from yellow spots. Bawden & Pirie (36) found cucurbit viruses 3 and 4 related to tobacco mosaic virus serologically. Knight & Stanley (37) confirmed this finding but report that they obtained weaker cross-precipitin reactions between the cucurbit viruses and tobacco mosaic virus than between cucurbit viruses 3 and 4. Serological reactions also showed that Holmes' ribgrass strain was less closely related to tobacco mosaic virus than to aucuba mosaic virus. In so far as they have been tested the different strains of tobacco mosaic virus found in nature and those obtained from yellow spots give protection against each other in appropriate hosts (15, 38 to 44).

Wyckoff (45) has reported that different strains of tobacco mo-

saic virus have widely different sedimentation constants. Pfankuch (46) and Knight & Stanley (47) found differences in the proportions or kinds of amino acids present. Knight and Stanley reported that the viruses of aucuba mosaic, green aucuba mosaic, Holmes' masked strain, and Jensen's 14-Dl strain contained the same proportions of tyrosine, tryptophane and phenylalanine, but that the virus of Holmes' ribgrass strain contained different proportions of each of these amino acids. Cucurbit mosaic viruses 3 and 4 also contained different proportions of tryptophane and phenylalanine. Knight (48) found 2 per cent of methionine in virus of the ribgrass strain but none in the viruses of seven other strains. The ribgrass strain also contained histidine which was not found in the viruses of eight other strains (49). It is interesting that the ribgrass and cucurbit 3 and 4 strains of tobacco mosaic virus that differ from the parent strain in amino acid composition also differ in host relationships. The ribgrass strain becomes systemic in ribgrass and in *Plantago major* (50, 51), but other strains remain local in these hosts. The cucurbit strains infect cucumber plants that are immune from all other tobacco mosaic virus strains.

Nothing is known regarding the origin of the ribgrass and cucurbit strains. Presumably they have been derived from the parent strain by mutations, but they may have arisen through a series of mutations rather than by single steps such as are believed to occur in bright yellow spots. Norval (30) has obtained and studied sixty-two variants from Jensen's 14-Dl strain of tobacco mosaic virus. Many of these submutants resembled some of the strains isolated by Jensen from yellow spots but one, at least, differed from any so obtained. Norval's No. 2 strain caused systemic necrosis in Turkish tobacco plants. It is somewhat similar to Jensen's 14-Dl strain which also is a submutant. Both strains caused systemic necrosis in tomato but only the Norval No. 2 strain produced generalized necrosis in tobacco. Norval found that many of the mutants derived from Jensen's 14-Dl strain were more severe on tobacco and tomato than the mutants derived by Jensen from ordinary tobacco mosaic virus. These cases suggest that wider variations may be obtained in two or more steps than in one. Strains of tobacco mosaic virus causing systemic necrosis in tomato (34) and tobacco (33) occur in nature but there is no information regarding their origin.

It is probable that some of the ways in which tobacco mosaic virus variants have been reported to differ from the parent strain

in tobacco and other plants are correlated. For example, rate of movement in plants may be correlated with rate of multiplication (52); ability to move out of inoculated leaves, with infectivity (30); and infectivity, with host nutrition (53, 54). Valleau & Diachun (52) found that different symptom complexes caused by different strains of tobacco mosaic virus could be duplicated by inoculating a single strain into tobacco plants having different degrees of resistance.

Kunkel (15) showed that in mutants from aucuba mosaic virus a factor for green mottling in tobacco and tomato varied independently of a factor for localizing in *Nicotiana sylvestris*. Holmes (55) found, in studies on mutants and submutants from a distorting type strain of tobacco mosaic, that factors for yellowing and invasiveness were independent of each other. Similar results were obtained by Norval (30) in studies on mutants from Jensen's 14-Dl strain of tobacco mosaic virus. His descriptions show that factors for necrosis, localizing, yellowing and distorting varied independently. In observations on frequency of occurrence of bright yellow spots in leaves of tobacco plants affected by tobacco mosaic virus of four different mottling type strains, derived from different sources, Kunkel (5) demonstrated that rate of production of bright yellow spots varied independently of a factor for green mottling possessed by all four strains. Under the same conditions the green mottling type strain derived from aucuba mosaic of tomato produced more than twice as many bright yellow spots and presumably more than twice as many yellow mosaic type virus mutants as did the green mottling strains derived from two different masked strains of tobacco mosaic virus, and about four times as many as did ordinary tobacco mosaic virus. This green mottling type strain from aucuba mosaic virus carried a stronger tendency for production of yellow type mutants than did the other green mottling strains that had been derived from green rather than yellow mosaic type viruses. Thus it appears that a factor for yellowing, which varies independently of several other factors in tobacco mosaic virus mutants and submutants, may be carried in a masked condition. Holmes (55) has suggested that factors which vary independently may represent unit differences in the structure of tobacco mosaic virus similar to unit differences in the genetic structures of plants and animals.

Cucumber mosaic strains.—Although possessed of different properties, ordinary tobacco mosaic virus and ordinary cucumber

mosaic virus have many hosts in common. Both produce green mottling symptoms in tobacco and other plants. If tobacco plants that have had cucumber mosaic for a month or longer are observed carefully it usually will be found that bright yellow spots occur on certain leaves. Though less numerous the spots are similar in size, shape and brightness to those which occur on leaves of tobacco plants affected by ordinary tobacco mosaic. From such spots Price (6) isolated fourteen yellow mosaic and necrotic type strains of virus. Five that were studied in several host plants were found to differ from each other but appeared to be closely related to cucumber mosaic virus from which they apparently arose by mutations.

A considerable number of different strains of cucumber mosaic virus occur on cucumber and other plants in nature (56). At least a dozen different strains have been observed on species in the Cucurbitaceae (3, 6, 57 to 63). Other strains have been isolated from banana (64), beans (65), cantaloupe (66), celery (67), *Chrysanthemum* (68), *Commelina* (69), *Delphinium* (70), hyacinth (71), lily (71, 72, 73), marrow (74), peas (65), *Primula obconica* (75), tobacco (76), tomato (60), tulip (71), and winter spinach (77).

All of the different strains that occur in nature or have been isolated experimentally have similar properties but differ strikingly in the severity of symptoms produced in different plants (56, 58, 78). Chester (13) showed that green and yellow mosaic type strains were closely related serologically, but experienced some difficulty in getting cross reactions with certain other strains (62). Price (79) developed a technique for making cross-immunity tests in plants of *Zinnia elegans* and showed that the immune reaction was specific for four different strains. The *Zinnia* immunization test later was used to show that celery mosaic and *Commelina* mosaic were strains of cucumber mosaic (67, 69). Two strains occurring naturally on peas and beans were found by Whipple & Walker (65, 80) to have slightly different thermal inactivation points and somewhat different host ranges. That a large number of different strains of cucumber mosaic virus are prevalent in nature, and that many yellow and necrotic type strains can be isolated experimentally from bright yellow spots on leaves affected by ordinary cucumber mosaic virus and in other ways, are well established facts. In general these results parallel those obtained with strains of tobacco mosaic virus.

Strains of latent mosaic of potato.—The latent or X-virus of

potato is another disease-producing entity that is represented in nature by many different strains. In so far as is known X-virus is prevalent wherever potatoes are grown. It can be isolated from almost all tubers produced commercially in the United States. However, in recent years an immune potato variety has been developed (81). Although X-virus is widely distributed and common, it is somewhat less prevalent in foreign countries than in our own (82). The strains have been divided into two groups,—a mottling type group and a ringspot type group (83, 84). Certain X-virus strains have been obtained experimentally by passage through different host plants (82). Böhme (85) working in Germany distinguished four different strains; Köhler (86, 87) obtained several others. Salaman (88) in England described six different variants. Four different groups were recognized in India (89). A new potato virus described by Bawden (90) and designated as D-virus was shown to be a strain of X-virus. Bawden & Sheffield (91) found that potato virus B also was a variant of X-virus. A mild disease of Royal Kidney potato in France was identified as a strain of the X-virus disease (92). A virus causing mottling and punctate necrotic lesions was found to belong in the potato ringspot group (93). A mosaic disease of the potato variety Mohawk was due to a severe mottling type strain (94). The mottling type group includes viruses that cause very mild symptoms or no symptoms at all. Strains in the ringspot group likewise differ considerably in severity. In tobacco and in potato also, no doubt, some strains move much more slowly (87) and some multiply more slowly than others (95). Certain strains have thermal inactivation points as high as 75°C., others as low as 68°C. (96). Mottling type strains are more thermostabile than the ringspot type strains (83). On the whole the different variants have similar properties, immunize against each other in suitable host plants (80, 83, 90, 92, 93, 94, 96, 97, 98) and give similar serological reactions (35, 83, 99). The masked strain does not always give complete protection against severe strains (100). Slow-moving strains give less protection than fast-moving strains (87). In plants inoculated simultaneously with strains of different severities a sufficient amount of virus of a mild strain will protect against a severe strain (88). Köhler (101) found that in tobacco a mild strain designated as Mb-12 protected against a more severe strain, H-19, when inoculations were made by the rubbing method, but not when a scion

infected by the mild strain was grafted onto a stock infected by the severe strain.

As might be expected of a parasite so widely distributed, the X-virus frequently occurs in plants affected by other viruses. In combination with tobacco mosaic virus in tomato it causes a severe streak disease (102). In combination with veinbanding virus in potato it causes rugose mosaic (103). Jones *et al.* (104) concluded that both the X-virus and the veinbanding virus are subject to such extensive modifications that in different combinations they are capable of causing a whole series of potato diseases including rugose mosaic, crinkle mosaic, mild mosaic, and various streak diseases.

Beet curly top strains.—Lackey showed that passage through *Chenopodium album*, tomato, squash, watermelon, spinach, and resistant varieties of sugar beets, gave attenuated strains of curly top virus. Passage of mild strains through the highly susceptible weed *Stellaria media* restored virulence (105); further work confirmed and extended these findings (106 to 109). Giddings (110) distinguished four different strains by their abilities to infect and injure different host plants. Later he isolated six other strains (111) and found that rate of spread in sugar beet fields depended on the strain of virus present (112). Bennett *et al.* (113) consider Argentine curly top of sugar beet to be a strain of the North American curly top, although it is transmitted by the leafhopper *Agalliana ensigera* and can not be transmitted by the sugar beet leafhopper, *Eutettix tenellus*, which spreads North American strains. They base their conclusions on similarities in host reactions, host ranges and properties of the two viruses.

The strains of curly top virus that have been tested generally have given little or no protection against each other. In tests on tomato Wallace (114, 115) showed that the degree of protection afforded depended on the strain of virus used. Giddings (116) found that infection of beets of a susceptible variety with his strains 2 and 4 did not give protection against his strains 1 and 3, but beets infected by strain 3 were resistant to infection by strain 1. Beets affected by virulent strains were subsequently infected by less virulent strains, and more virulent strains were readily introduced into plants affected by less virulent strains (117).

Sugar cane mosaic strains.—Storey (118) as early as 1923 reported that, in Natal, Uba cane which was generally considered

resistant to sugar cane mosaic virus, was affected by a mottling disease. Robertson (119) noted that sugar cane mosaic in Burma caused such slight damage as to suggest that it was different from the sugar cane mosaic of other countries. These observations suggested but did not prove that distinctive strains of sugar cane mosaic virus occurred in different sugar cane-growing countries. By 1928 Storey (120) recognized that two strains were prevalent in Natal. Since then many different variants have been found occurring naturally in different parts of the world. Ten different strains have been recognized in Louisiana (121), three in Jamaica (122), three in India (123), and two or more in Puerto Rico (124). According to Martin (125) strains present in Hawaii are of low virulence as compared with other known strains. Matz (126) states that strains prevalent in other regions of the Pacific also are of low virulence and are more difficult to transmit to certain varieties than are the Louisiana strains.

The different variants of sugar cane mosaic virus are distinguished by differences in distribution, symptoms, varieties attacked (122), thermal death points, longevity, extent of recovery permitted (123) and reduction of yield (127). A severe strain in the variety C.P.28/70 reduced tonnage by about one-third and sucrose content by 20 to 30 per cent, but a mild strain caused very little reduction in growth and only slight reduction in sucrose (127). One green mosaic type strain gave protection against a severe green mosaic and a yellow mosaic type strain, but another from the variety Louisiana Purple gave no protection against either (128).

Other plant virus strains.—Strains of many other virus diseases have been reported. Some have been studied carefully and others very little. Mild and severe strains of etch disease occur on tobacco in this country (59) and in England (129). Several different tobacco ringspot strains are prevalent in Kentucky (130) and three are said to cause yellow spotting of potato foliage in Germany (131). McClean (132) states that leaf curl of tobacco, of which there are many strains, and curly top of sugar beets resemble each other and should be classified close together. Although spread by different vectors, Black (133) considers New York yellow dwarf and New Jersey yellow dwarf of potato to be strains of one disease. The viruses causing Canada streak, pseudo-net necrosis and aucuba mosaic of potato are believed to be related (134). Studies on serological and physical properties of veinbanding, stipple streak, and Y viruses of potato indicate that they belong in one group (88).

Unmottled curly dwarf of potato seems to be caused by a strain of the spindle tuber virus (135). Potato calico virus is a strain of alfalfa mosaic virus (136). A California strain of aster yellows infected celery plants that were immune from the New York strain (137). Mild strains of aster yellows were transmitted by viruliferous aster leafhoppers that were held at 32°C. for a few days (138). Younkin (139) reported that four strains of aster yellows from potato differed from the strain prevalent on *Ambrosia*. Cabbage virus A, black ring virus, and ring necrosis virus (140) of cabbage are believed to be strains of turnip mosaic virus. Cabbage virus B apparently is a strain of cauliflower mosaic virus (141). Eight strains of the cacao swollen shoot disease have been recognized (142). There is evidence that spotted wilt of tomato is a complex of related strains (143). Marginal leafspot of peach probably is a strain of western X-disease (144) and western X-disease probably is a strain of the eastern form (145). There are so many different variants of peach mosaic virus that it is difficult to recognize individual strains (146). Little peach and peach yellows seem closely related (147). Other strains or suspected strains of plant viruses have been listed (148).

Discussion

Research data show that there are some viruses with large and some with small families of variants. Tobacco and cucumber mosaic viruses belong in the large family group, aster yellows and peach yellows viruses in the small family group. There also are a few like the viruses of peach rosette and potato leaf roll that seem to produce no variants. They at least have not given rise to recognizable strains. Rosette virus has a limited distribution and has not been studied intensively, but leaf roll virus is practically world wide in distribution and has been the object of many researches. If it produces mutants they should have been seen and reported. Both viruses parasitize hosts in which other viruses produce large numbers of mutants; hence, their failure to mutate cannot be attributed to host environment. Whether these or any other plant viruses are so stabile that they never mutate is not known, but that there are great differences in the mutabilities of viruses seems certain.

Rate and extent of variability seem to be specific characteristics of many plant viruses. Numerous strains occur naturally and additional strains may be obtained experimentally. One of the best

ways of securing variant strains is to isolate from bright yellow spots that occasionally appear on leaves of plants affected by such diseases as tobacco mosaic and cucumber mosaic. These isolations yield yellow and necrotic type strains. It is believed that other types of virus strains also occur locally in diseased plants but do not produce local effects that can be seen. Isolation of strains by direct inoculation from bright yellow spots has been limited to viruses that can be transmitted mechanically by means of juice, but it would seem that the feeding of insects, either by accident or through confinement on areas where mutants have occurred locally, might isolate strains not mechanically transmissible. Another good way of obtaining strains experimentally is to pass viruses in different host plants. The strain that flourishes in one variety or species may not be the strain that will do best in another. Hence, if a virus is passed in a host other than that in which it predominates, it may be replaced by one or another of the mutants that arise from time to time. Occasionally a new strain may be secured by one passage but more often a series of passages is needed. It usually is not possible to predict with any degree of certainty the number of passages that will be required because it always is necessary to await the production of a suitable mutant. Variant strains also have been secured by holding diseased plants or specific insect vectors at high temperatures. Success with this method seems to depend on the fact that some virus strains multiply at higher temperatures than others. Variant strains also may be isolated by plating out a virus such as that of tobacco mosaic on a host in which conspicuous local lesions are produced, and then subinoculating from individual lesions to one or more hosts in which new strains can be differentiated.

Variant strains of plant viruses have been shown to differ from parent strains in a variety of ways including symptoms, severity of disease produced, infectivity, longevity, dilution end point, thermal inactivation point, amounts and kinds of amino acids, temperature range for multiplication, invasiveness, rate of movement in tissues, extent to which they become systemic, types of primary and secondary lesions, geographical distribution, rate of spread, effect on yield, host range, specific insect vectors, mutabilities, serological and immunological reactions.

Viruses frequently have been compared with the genes of higher plants. Viruses and genes are said to be of the same order of size. Both mutate and both appear to be composed chiefly of nucleoproteins. Moreover, they both reproduce only in living cells.

But whereas multiplication of genes ordinarily is no faster than rates of nuclear divisions, viruses far exceed those rates. Also, viruses pass from cell to cell, while genes do not, except during fertilization. Viruses are autonomous; genes are a part of the hereditary mechanism. A gene represents a single factor in reproduction and may be of simple structure. A virus like that of tobacco mosaic, which has been shown to carry a series of factors capable of varying independently in mutations, must possess sufficient organization to permit such variability. In this respect tobacco mosaic virus behaves more like a chromosome than a gene.

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SOME ASPECTS OF THE PROBLEM OF GROWTH FACTORS FOR PROTOZOA

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The phylum Protozoa comprises an assembly of forms extremely diverse both from the morphological and physiological viewpoint. The Protozoa are "noncellular" organisms possessing a distinct nucleus. They can only be defined by exclusion: they are neither fungi, nor algae, nor bacteria. Their boundaries in respect to the lower Metazoa can be rather clearly traced: the most highly differentiated protozoa, the Ciliates, are easily separated from the simplest forms of invertebrates.

On the other hand their boundaries with the protophytes are uncertain, in fact nonexistent. If one considers that certain phytoflagellates may lose their chlorophyll or their chlorophyll and their plastid; may lose the power to synthesize starch or related substances; may acquire amoeboid movement; may combine photosynthesis and phagocytic nutrition; and finally that certain types may, during the course of a phylogenetic evolution whose stages can be followed step by step, permanently lose all the attributes of the plant kingdom, one can easily see that attempts to establish clear-cut limits between "plant like" and "animal like" protista are futile and meaningless. It is no more possible to define the Protozoa, than it is possible to give a satisfactory differential diagnosis between plant and animal. The diversity of characteristics explains the need for a study of the comparative physiology of the nutrition of "protozoa" in the broad sense of the word.

The definition of trophic requirements can only be established with pure cultures. One of the reasons for the late development of studies on the comparative physiology of protozoa has been the lack of protozoological knowledge by most bacteriologists, and a deficiency of bacteriological and biochemical knowledge by most protozoologists. But during the past few years, a current of interest in this field has been apparent, influenced by the recent discoveries in relation to vitamins and to bacterial nutrition. The importance of protozoa in pathology and in war time problems, and the advances in rational chemotherapy have also stimulated scientists to studies in this domain.

Various phenomena—encystment, excystment, sexuality, determinism of phases in the evolutionary cycle—are all based on trophic phenomena.

The interest attached to the study of the growth factors needed by protozoa is obvious. An exact knowledge of growth factors is essential for the study of protozoa in chemically defined media and therefore for the solution of numerous problems in the fields of pure and applied biology. To-day, the protozoa are no longer the poor relations of microbial chemistry, and one can forsee a rapid advance in our knowledge of their comparative physiology.

In recent years, considerable progress has been made in studies on the problem of the synthesizing ability of protozoa. A number of essential metabolites which protozoa are incapable of synthesizing have been found, i.e., a number of vitamin or growth factor requirements have been identified. On the whole, in regards to vitamin requirements, there seems to be a parallelism between the various types of protozoa, and the various types of other microorganisms: yeasts, molds and bacteria. However the protozoa seem to possess a monopoly of certain growth factors.

Several articles have already been devoted to the discussion of these problems. Specifically, certain chapters in *l'Evolution physiologique* (1); a monograph on the *Pouvoir de synthèse des Trypanosomides* (2); and two reviews, one of which deals with the general aspects of nutrition of protozoa (3), and the second concerned only with growth factors (4), have appeared between 1940 and 1943. In addition, a well-documented book describing the relationships between plants and vitamins (5), and a very comprehensive review discussing the growth factors required by bacteria (6) have been published recently. It is not therefore our intention to discuss the whole question of growth factors for protozoa once more.

Since 1943, many problems have been attacked, and some solved. The Harvard group (7) has succeeded in growing malarial parasites *in vitro*. Their complete studies have not yet been published. From their preliminary publications, it appears that *p*-aminobenzoic acid is one of the growth factors required by *Plasmodium knowlesi*.

Granik & Gilder (8) have recently studied the activity of various hematin derivatives as growth factors for *Haemophilus influenzae*. Some derivatives which were inactive for Trypanosomes

[see M. Lwoff (2)] were active for *Haemophilus*, while others exhibited an inhibitory action. These studies present new data on the physiological function of hematin and its biosynthesis, which should be applied to studies with Trypanosomes.

During the past few years, Kidder & Dewey (9 to 12) have succeeded in cultivating some ciliates in media of known composition. In these studies they identified the indispensable amino acids and the growth factors of the vitamin B complex required for the growth of particular ciliates. Kidder and Dewey reach the conclusion that thiamine which is not synthesized by *Tetrahymena piriformis* (=*geleii*) from pyrimidine and thiazole in natural media, is entirely synthesized in the presence of any one of the nonnatural amino acids.

We would have liked to review all these papers adequately but unfortunately, in France, our collections of scientific journals are still incomplete, and this review cannot hope to present a complete picture of the studies which have appeared between 1943 and 1947. We have therefore been forced to limit ourselves to the discussion of the question of the thiamine requirements of some colorless and chlorophyll bearing flagellates.

As will be seen in this review, conflicting results have been obtained. And this calls for a remark which seems to us of great importance. It is understandable that, up to a certain point, different authors obtain different results in dealing with the same species. But it is regrettable that the results cannot be repeated because of inability to obtain the strain used. The following example shows the confusion that can exist because of the absence of a collection of type cultures.

Among the varieties of flagellates studied by Ondratschek (13 to 20) one finds almost the complete list of known species of *Chilomonas*, *Polytomella* and *Polytoma*, including the celebrated *Polytoma Pascheri*, described by Moewus (21) who studied the genetics of two remarkable varieties. These two varieties were crossbred. A considerable number of descendants were studied and combinations of the characteristics of the two varieties were observed. It is highly desirable that these results be analyzed statistically. Actually, such an analysis applied by Philip & Haldane (22) to other experiments of Moewus, has led to a conclusion which is extremely critical of Moewus's work.

It would be very worthwhile to be able to locate *Polytoma*

Pascheri. Unfortunately, a few years ago, an agreement with Moewus did not lead to the results anticipated. In exchange for my strains, Moewus was not able to send me *P. Pascheri* which, at that moment, refused to grow. Moewus also obtained the hybrids of the two varieties of *P. Pascheri* and of two varieties of *P. uvella* in the medium utilized for the growth of *P. Pascheri* at pH 4.5. In this medium, Moewus was able to obtain growth of his two varieties of *P. uvella.* Now there is one point on which all who are in the habit of cultivating *Polytoma* are in agreement: *P. uvella* does not grow when the pH is below 7.0. Consequently, it is to be feared that some difficulty might be encountered in trying to cultivate again these two varieties of *P. uvella* which are really so convenient for genetical work. In addition, one should note that while Moewus claims to have been able to cultivate *P. Pascheri* at pH 4.5, Ondratschek (18) affirms that this species does not develop below pH 6.5. *Polytoma Pascheri* remains clothed in mystery!

Now, it is also highly probable that considerable difficulties might be encountered by anyone wishing to cultivate the two varieties of *P. Pascheri* described by Moewus. For no one else, with the exception of Ondratschek has ever had the occasion to see *P. Pascheri.* After the liberation of Prag, I tried, in vain, to get in touch with "Reinhardt", the new name adopted by Ondratschek in 1943 (20). I was told that Ondratschek-Reinhardt had disappeared from Prag, and his cultures were not to be found at the Botanical Institute.

It would be desirable if authors, who publish an experimental study on a determined species, strain or clone, were required to deposit a culture in a collection of type cultures. It is to be feared that lacking this precaution, the scientific literature will find itself burdened with experiments which cannot be verified due to the "loss" of certain strains because of unforseen and most unfortunate, or fortunate, circumstances.

Are Growth Factors Needed by *Polytoma Uvella* and *Polytoma Obtusum*?

The answer to this question seems simple. All one needs to do is to inoculate the flagellates into a medium devoid of growth factors, and note whether or not growth occurs. However Lwoff & Dusi (23 to 28) on one hand and Ondratschek on the other (16, 17, 18, 20) have obtained completely different results. These diver-

gences could be attributed to differences in technique, so let us examine the methods employed.

TABLE I

THE INFLUENCE OF THIAMINE AND OF IRON ON THE GROWTH OF CERTAIN FLAGELLATES

[unpublished data of A. Lwoff]

Added constituents	*Polytoma uvella*		*Polytoma obtusum*		*Polytoma ocellatum*		*Polytomella caeca*	
Thiamine	—	M/3.5 $\times 10^{-8}$	—	M/3.5 $\times 10^{-8}$	—	M/3.5 $\times 10^{-8}$	—	M/3.5 $\times 10^{-8}$
Control culture	0	0	0	0	0	0	0	0
Ferrous sulfate*								
M/5 $\times 10^{-3}$	+[3]	+	+[3]	+	0	+	0	+
Hemine								
M/1 to/6 $\times 10^{-5}$	+†[3]	+‡	0	0	0	0	0	+
Ferric citrate								
M/3 $\times 10^{-4}$	+	+	+	+	0	+	0	+
M/15 $\times 10^{-4}$	+	+‡	+	+‡	0	+	0	+
M/3 $\times 10^{-5}$	±†	+	±†	+	0	+	0	+
M/6 $\times 10^{-5}$	±†	+	±†	+	0	±	0	+
M/15 $\times 10^{-5}$	±†	+	0	±	0	0	0	+
M/3 $\times 10^{-6}$	±†	+	0	0	0	0	0	+
M/3 $\times 10^{-7}$			0	0	0	0	0	+†
Na_2S or H_2S								
M/800	+[2]	+[2]	0	0	0	+[2]	0	+[3]

* Added after inoculation.

† Slow development or long lag phase.

‡ Thiamine accelerates growth.

0 = No growth of culture.

+ = Good growth, 800 to 1,200 flagellates per c.mm.

± = Irregular growth, either positive or negative.

[ ] = Figures in brackets indicate maximum number of subcultures.

Lwoff and Dusi, first used media containing asparagine, and later, media containing ammonium acetate. Their culture tubes of glass (pyrex) were capped with glass caps with or without a side-arm. Sterility was guaranted by an asbestos plug (previously heated red-hot) placed between two glass wool plugs. Two flagellates *Polytoma ocellatum* and *Polytoma caeca*, the former requiring thiazole and the latter both thiazole and pyrimidine were used as controls. In the asparagine medium, these control flagellates give very poor cultures, 3 to 14 flagellates per c. mm. With thiamine added to the medium, growth was abundant: 800 to 1200 flagellates per c.mm. In the ammonium acetate medium these cultures were lost, regularly, at the second transplant.

Table I shows that *P. uvella* and *P. obtusum* developed in a synthetic medium [0.75 gm. $(NH_4)_2SO_4$, 1.0 gm. CH_3COONa, 0.05 gm. $MgSO_4$, 0.25 gm. KH_2PO_4, 0.01 gm. $CaCl_2$, and NaOH to pH 6.0 to 7.4 according to the species, per liter of redistilled water] in the absence of added thiamine when an acceptable source of iron at an adequate concentration was provided. The inoculum per 5 cc. of culture medium for *P. uvella* and *P. obtusum* consisted of two drops of a culture grown with subminimal amounts of iron, for *P. ocellatum* and *P. caeca* two drops of a culture grown with subminimal amounts of iron and of thiamine. *P. uvella* utilized ferrous sulfate, ferric citrate and hemine; *P. obtusum* utilized ferrous sulfate and ferric citrate only. But the ferrous sulfate was active only if added after sterilization, or preferably, after inoculation with the flagellates.

One notes, that with certain concentrations of ferric citrate, the addition of thiamine produced a noticable accelerating action on growth. With other concentrations, the initiation of growth was very slow and thiamine no longer acted as an accelerator. Finally, with low concentrations of iron, the results were irregular, sometimes growth occurred, sometimes no culture was obtainable, while in the presence of thiamine growth appeared slowly.

This indicates that there are certain concentrations of iron at which growth will not take place except in the presence of thiamine. Now, Ondratschek used media at pH 7.5 in which ferric chloride was added before sterilization at a concentration of $M/1.6\times10^{-5}$. Everyone knows, for the reaction is clearly visible, that the iron in ferric chloride precipitates in alkaline media. The real concentration of iron in Ondratschek's media was therefore notably inferior to $M/1.6\times10^{-5}$, and it is probably for this reason that thiamine was found to be indispensable in this media. One can see the importance of a source of soluble iron for the culture of these flagellates.

Table I also shows that Na_2S or H_2S permitted some growth of *P. uvella* (one or two transplants only), but not of *P. obtusum* or *P. ocellatum*. On the other hand, *P. caeca* could be cultured during three serial transplants in media without iron if supplemented with either Na_2S or H_2S, substances which lower the redox potential of the media. Whereas, according to Ondratschek, *P. uvella* and *P. obtusum* showed no development whatsoever in the absence of thiamine. The optimum concentration of thiamine for *P. uvella*

was 1×10^{-11} and thiamine still showed some activity at 1×10^{-15}. According to our calculation 2,100 molecules per c.mm. would permit a development of about forty flagellates or only one-thirtieth as many as obtained (1,200) with an optimum concentration of thiamine which is 10,000 times stronger! These results require very careful consideration.

It has been generally observed that the action of growth factors is, within certain limits, proportional to the concentration. In the experiments of Ondratschek, the number of flagellates approximately doubled when the concentration of thiamine was increased ten times.

There are other equally interesting results in Ondratschek's work. It is known that for all microorganisms, growth, within certain limits, is proportional to the amount of the nitrogen source. But according to Ondratschek, the growth of *P. uvella* is doubled when the concentration of the nitrogen source is increased ten times. Rottier (29, 30) has carefully demonstrated that the development of *P. uvella* is strictly proportional to the concentration of the nitrogen source provided the cultures be adequately aerated: the number of flagellates can then approximate 12,000 per c.mm. The media used by Ondratschek was divided into 250 cc. portions in 300 cc. Erlenmeyer flasks. The semi-anaerobic state in which his flagellates developed can explain his aberrant results. These are not confined to *P. uvella*. All the flagellates studied by Ondratschek show the same lack of proportionality between thiamine or nitrogen concentration and development.

We should add that *Polytoma curvatum*, *P. Pascheri* and *P. obtusum* which according to Ondratschek do not grow in the absence of thiamine, produce abundant cultures in the presence of either thiazole or pyrimidine. This is really a very interesting observation which we would like to see repeated. At any rate all these studies should be verified under experimental conditions which would permit one to observe a proportionality between growth factors or nitrogen concentration and the growth of the flagellates.

We consider it to be probable that *Polytoma uvella* and *Polytoma obtusum* can develop without an exogenous source of thiamine; that certain species such as *Polytoma ocellatum* require thiazole alone; others, like *Polytomella caeca* (23) and *Chilomonas paramoecium* (26) need thiazole and pyrimidine.

TABLE II

RELATIVE ACTIVITY* OF THIAZOLE SUBSTITUTES
[from results of Lwoff and Dusi]

—Thiazole	2	4	5	*Polytoma ocellatum*	*Polytoma caudatum*	*Polytomella*† *caeca*	*Chilomonas*† *paramoecium*
4-methyl	H	CH_3	H	0	0	0	0
4-5-dimethyl	H	CH_3	CH_3	0	0	0	0
4-methyl-5-vinyl	H	CH_3	$CH \cdot CH_2$	0.05	0.5	0.05	
4-methyl-5-β-aminopropionic	H	CH_3	$CH_2 \cdot CHNH_2 \cdot COOH$	10		10	
4-methyl-5-α-hydroxyethyl	H	CH_3	$CHOH \cdot CH_3$	0	0	0	
4-methyl-5-ethyl	H	CH_3	$CH_2 \cdot CH_3$	0	0	0	
2-hydroxy-4-methyl-5-acetoxyethyl	OH	CH_3	$CH_2 \cdot CH_2O \cdot COCH_3$	0	0	0	
2-amino-4-methyl-5-β-hydroxyethyl	NH_2	CH_3	$CH_2 \cdot CH_2 \cdot OH$	0	0	0	
2-iodomethyl-5-methyl-5-β-hydroxyethyl	ICH_2	CH_3	$CH_2 \cdot CH_2 \cdot OH$	10		1	
2-4-dimethyl-5-β-hydroxyethyl	CH_3	CH_3	$CH_2 \cdot CH_2 \cdot OH$	1,000	1,000	1,000	
4-methyl-5-β-hydroxyethyl	H	CH_3	$CH_2 \cdot CH_2 \cdot OH$	1,000	1,000	1,000	1,000
4-methyl-5-acetoxyethyl	H	CH_3	$CH_2 \cdot CH_2O \cdot COCH_3$	100	1,000	100	100
4-methyl-5-β-hydroxypropyl	H	CH_3	$CH_2 \cdot CHOH \cdot CH_3$	1	1	1	
4-methyl-5-γ-hydroxypropyl	H	CH_3	$CH_2 \cdot CH_2 \cdot CH_2OH$	0.1	1	0.1	
4-methyl-5-β-aminoethyl (picrate)	H	CH_3	$CH_2CH_2NH_2$ (picrate)	100		100	
Thiamine				1,000	1,000	1,000	1,000
Thiochrome				20	0	0	5

* Relative activity in comparison with thiamine. The highest dilution (active limit) of thiamine supporting small but definite growth of *P. ocellatum* and of *P. caeca*, 3×10^{-10} M., and *P. caudatum* and of *C. paramoceium*, 3×10^{-9} M.

If substitutes are active at the active limit, their activity is 1,000. If the limit of action is a concentration 100× that of thiamine, the activity would be 10, etc.

† Growth in the presence of an excess of 2-methyl-4-amino-5-aminomethylpyrimidine

0 = Substituted thiazole does not support growth.

Because of the divergencies which exists between the results and conclusions of various authors, the problem of the growth factors needed by *Polytoma*, *Polytomella* and *Chilomonas* species undoubtedly requires further study.

Specificity of Thiamine Constituents for Flagellates

As the data illustrating the activity of various thiazole and pyrimidine derivatives has not been presented in schematic form we believe it might be useful to present tables summarizing the

TABLE III

Activity* of Pyrimidine Substitutes
[from results of Lwoff and Dusi]

—Pyrimidine	4	5	*Polytomella caeca*	*Chilomonas paramoecium*
2-methyl-4-amino-5-aminomethyl	NH	CH_2NH_2	2×10^{-10}	2×10^{-10}
2-methyl-4-amino-5-bromomethyl	NH_2	CH_2Br_2	2×10^{-10}	2×10^{-10}
2-methyl-4-amino-5-hydroxymethyl	NH_2	CH_2OH	1×10^{-10}	3×10^{-10}
2-methyl-4-amino-5-thioformyl-aminomethyl	NH_2	CH_2NHCSH	6.6×10^{-9}	6.6×10^{-9}
2-methyl-4-amino-5-acetamid	NH_2	CH_2CONH_2	0	0
2-methyl-4-amino-5-carboxy	NH_2	COOH	0**	
2-methyl-4-amino-5-carbamyl	NH_2	$CONH_2$	0**	
2-methyl-4-amino-5-methanal	NH_2	CHO	+**	
2-methyl-4-amino-5-cyano	NH_2	CN	+s**	
2-methyl-4-amino-5-methyl	NH_2	CH_3	0	0
2-methyl-4-amino	NH_2	H	0	0
2-methyl-4-hydroxy-5-aminomethyl	OH	CH_2NH_2	0	0
2-methyl-4-hydroxy-5-hydroxymethyl	OH	CH_2OH	0	0
2-methyl-4-methoxy-5-aminomethyl	OCH_3	CH_2NH_2	0	0
2-methyl-4-hydroxy-5-amino-6-methyl	OH	NH_2	0	0
4-amino-5-methyl-Th.‡-6-methyl	NH_2	CH_3-TH.‡	0	0
Thiamine			2×10^{-10}	2×10^{-10}
Thiochrome			4×10^{-9}	4×10^{-9}

* Expressed as highest molar dilution supporting visible growth.

** Results from Javillier and Emerique-Blum.

‡ Th. = 4-methyl-5-β-hydroxyethyl thiazole (the pyrimidine is combined in the form of isovitamin B_1).

0 = Does not support growth in concentration as great as 6×10^{-6} M.

\+ = Supports growth, no quantitative data.

\+s = Slow growth.

† Growth in the presence of an excess of 2-methyl-4-amino-5-aminomethylpyrimidine
0 = Substituted thiazole does not support growth.

results of Lwoff & Dusi (27, 31, 32), and those of Javillier & Emerique-Blum (33, 34). This presentation facilitates the comparison between flagellates on the one hand, and bacteria and fungi on the other.

Necessity of Growth Factors for Phototrophic Organisms

The dogma of mineral nutrition for chlorophyll bearing organ-

isms has long dominated the study of physiology. It was understood that photosynthetic organisms were autotrophic, meaning that they could synthesize all their constituents from minerals. However, in 1930 Dusi (35) showed that certain species of Euglena: *E. deses*, *E. pisciformis* and *E. anaboenea* could not multiply in the absence of organic substances. Note well that these flagellates can only develop in the presence of light: they are obligate phototrophs.

Thus we were forced to separate two nutritional phenomena hitherto considered indissolubly united: the photosynthetic assimilation of carbon and a purely mineral nutrition. Photosynthesis

TABLE IV

INFLUENCE OF THIAMINE AND OF PYRIMIDINE ON THE GROWTH OF *Euglena pisciformis* IN THE LIGHT
[from results of Dusi (36)]

Subculture	B.M.	+B_1	+Th.	+Py.	+Py.+Th.
	Number of flagellates per c.mm.				
1	100–150	100–150	100–150	100–150	100–150
2	10	100–150	8	11	100–150
3	0	100–150	0	0	100–150
12	0	100–150	0	0	100–150

BM = Basic medium, 2.0 gm. asparagine (thrice recrystallized), 0.1 gm. $MgSO_4$, 0.2 gm. KH_2PO_4, 0.1 KCl per l. redistilled water, NaOH to pH 6.8 to 7.5, 0.01 gm. $CaCl_2$ and 0.01 gm. iron citrate added after sterilization.
+B_1 = Basic medium plus thiamine
+Th. = Basic medium plus 4-methyl-5-β-hydroxyethyl thiazole
+Py. = Basic medium plus 2-methyl-4-amino-5-aminomethyl pyrimidine

could be connected with the need for organic substances, and an organism could be simultaneously phototrophic and heterotrophic.

Euglena gracilis, a facultative phototroph, could be grown in darkness if provided a suitable carbon and energy source, for example, fatty acids. But while this species normally grows in a mineral medium, it cannot grow in the absence of light and organic substances. One of the indispensable compounds for growth was identified by Lwoff & Dusi (26) as 2-methyl-4-amino-5-aminomethyl pyrimidine. Subsequently, Dusi (36) showed that the growth factors required by *E. pisciformis*, which is an obligate

phototroph, were pyrimidine and thiazole. And so the dogma of mineral nutrition for chlorophyll bearing organisms was shattered.

The above studies seem to have been completely overlooked by Ondratschek who rediscovered later (19) that thiamine was needed by certain Euglenas: *E. gracilis* and *E. viridis*, *Astasia longa*, *A. quartana* and *A. chattoni*. These species grew only in the presence of thiamine or thiazole. Ondratschek carefully indicated that all his experiments were carried out in the dark.

However, according to the observations of Dusi (37, 38) *Euglena viridis* is incapable of growing in the absence of light. And thiamine in the presence of light exerted only a mild stimulatory action even with the addition of various oligoelements, (zinc, manganese, etc. . . .).

Naturally, differences between various strains may exist, but at any rate, all attempts by Dusi to repeat the studies of Ondratschek have failed. On Ondratschek's medium, supplemented with thiamine, zinc and manganese in the optimum concentrations indicated by the German author, Dusi has been unable to obtain serial development of *Astasia Chattoni*. Where Ondratschek obtained abundant cultures, Dusi (unpublished results) obtained no growth at all, after the peptone from the stock culture had been eliminated by serial transfer.

Other experiments of Ondratschek could not be repeated. *Haematococcus pluvialis*, in darkness, in a medium containing asparagine plus sodium acetate gave cultures varying from 100 to 200 flagellates per c.mm. According to Ondratschek (14) the addition of thiamine or ascorbic acid produced cultures yielding about 4,800 flagellates per c.mm.

Those who have studied *Haematococcus* (a relatively voluminous flagellate) will undoubtedly be surprised at the abundant cultures obtained by Ondratschek in nonaerated test tubes. Dusi (in his unpublished results) has found that thiamine produces only a very slight—if any—increase in growth.

We may admit that the need for thiazole and pyrimidine by *Euglena pisciformis* has been demonstrated. On the other hand it seems equally certain that thiamine is not the sole growth factor for *Euglena gracilis* grown in the absence of light, and for *Astasia Chattoni*. The problem of growth factor requirements for chlorophyll-bearing or chlorophyll-free *Euglenides* should be taken up experimentally again.

Under all circumstances, one fact remains certain, some chlorophyll bearing, obligate phototrophic flagellates, require growth factors among which pyrimidine and thiazole have been identified. And it is interesting to observe that the need for growth factors is not only a requisite of photosynthetic flagellates, but exists among certain cholorophyll containing bacteria as well. Hutner (39) was able to demonstrate that biotin, thiamine and nicotinic acid were among the growth factors required by certain species of *Rhodospirillum*.

On the whole, the problem of the growth factor requirements of the few flagellates examined is not entirely resolved. Agreement has been reached on one point: the importance of thiamine constituents for their nutrition. The need for thiazole and pyrimidine is therefore not monopolized by bacteria, fungi and cells of certain higher plants. It is found among the *Cryptomonadina*, the *Phytomonadina* and the *Euglenidina*. The loss of the power to synthesize thiazole and pyrimidine thus appears to be one of the earliest and most constant symptoms of physiological evolution.

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BACTERIAL METABOLISM

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In 1943, van Niel (1) noted a general shift of emphasis in this field from general and intermediary metabolism towards studies centered around bacterial nutrition. This trend has been very marked during the present year, and, although "growth factors" were the subject of a separate article by Snell (2) last year, there is again much to report. On the other hand there have been comparatively few studies dealing with the end products and intermediate stages of catabolic processes. Detailed consideration of this aspect will therefore be omitted this year since it was covered fully by Barker & Doudoroff (3) in 1946. Furthermore, the more important advances in recent years have been made by the use of isotopic "tracer" elements, and an article by Kamen on this method will be found elsewhere in this volume.[1] Two general reviews (4, 5) include work on the fixation of carbon dioxide by bacteria, and Lipmann (6) has summarized the role of acylphosphates in bacterial metabolism.

Inhibition of growth by substances chemically related to essential metabolites remains an active field of research. The subject has been recently reviewed comprehensively by Roblin (7), and in more general terms by Woolley (8), and will not be dealt with here except when such inhibitors have proved useful tools in particular researches. General aspects of bacterial growth are considered in monographs by Monod (9) and, from the physicochemical standpoint, by Hinshelwood (10). The biological background of our subject is emphasized in Dubos' (11) excellent book. The biochemical activities of marine microorganisms have been surveyed by ZoBell (12).

There can, of course, be no real distinction between metabolism and nutrition (though the terms are often used for convenience in a general sense), and this is exemplified by recent progress (reviewed below) towards an understanding of the precise function of several growth factors. An outstanding problem in all branches of biochemistry is that of the anabolic processes of the cell, but an

[1] *Ann. Rev. Biochem.*, 16, 631–54 (1947).

encouraging beginning has been made with microorganisms, mainly with regard to the nature of intermediate compounds in the synthesis of essential metabolites.

Analysis of the ultimate details of enzyme reactions usually involves the use of isolated enzyme systems. Utter *et al.* (13) have published some helpful general observations concerning their glass-grinding technique of preparing cellfree bacterial extracts. Stumpf & Green (14) give similar information respecting their ultrasonic disintegration procedure. This method, and that suggested (15) by Curran & Evans (shaking with minute glass beads) have not yet been much used in enzyme work but should prove valuable. An observation which may have wider application has been made by Utter *et al.* (16) who find that salts increase the range of substrates oxidized by cells lysed with lysozyme.

Bacteria occur naturally in mixed populations rather than as pure cultures, and more detailed knowledge of their biochemical activities under these conditions is much needed. Recent examples of this type of work include the study, by a new technique, of nitrification in soil (17), and of carbohydrate metabolism in the sheep rumen (18).

As in previous years it has been necessary to restrict the detailed portion of this review to certain topics. The material has been chosen to illustrate general methods of investigation in those fields and it has, of course, been impossible to include every publication.

Nutrition

Work concerning individual growth factors was reviewed last year (2) and the present review will be concentrated on more general aspects. Lwoff (19) has amplified his views on the relationship of growth factor requirements to evolution amongst microorganisms. Other reviews deal with the utilization of hydrocarbons (20) and the nutrition of lactic acid bacteria (21). Dubos' (22) important observation that rapid, diffuse and abundant growth of tubercle bacilli can be obtained by the addition of surface active substances to the medium should facilitate studies with these organisms. The problem of the obligate anaerobe has again been tackled (23 to 25); synthetic reactions appear to be inhibited by oxygen whilst energy yielding reactions are not.

The importance of the inorganic constituents of a medium has been emphasized by several studies. The correct balance of inor-

ganic salts greatly improves growth and spore yield of the anthrax bacillus on a synthetic medium (26). When the salt concentration of the normal medium is reduced, the ability of *L. fermenti* to synthesize histidine is much increased (27). Photosynthetic purple bacteria require some unusual trace elements (28) which have not yet been fully defined; there are great technical difficulties in this work.

The requirements of several new groups of organisms have been examined. Photosynthetic purple bacteria are able to live autotrophically except for the need of complex microfactors. Hutner (28) has identified these factors as members of the vitamin B group; there is considerable species specificity as to the factors required and several unclassified strains were assigned to species on this basis. Plant pathogens of the genera *Xanthomonas* and *Agrobacterium* will often grow on simple media but some strains require amino acids and/or B vitamins (29).

High yields of fully virulent anthrax spores have been obtained in a chemically defined medium (26); the criterion used throughout was spore yield, so it is not possible to say if there are special requirements for spore formation as opposed to vegetative growth. It is thought that the amino acid mixture finally used might be improved since there is a relation between optimum amino acid nutrition and the concentration of other components of the medium. Thiamine is an absolute requirement, while purines and glutamine are stimulatory. The amino acid and growth factor nutrition of *Cl. botulinum* has also been worked out in detail (30); here biotin is the only essential factor though other B-vitamins and nucleic acid derivatives improve growth. The essential amino acid requirements of *Strept. fecalis* (31) and *L. fementi* (27) have also been reported. Cystine is the best form of sulphur for *Proteus morganii*, though several other compounds, including sodium sulphides, permit fair growth (32).

Acetate has been commonly used as a buffer in synthetic media for *Lactobacilli* and other organisms. Guirard *et al.* (33) have now shown that it has a specific function in stimulating initiation and rate of growth. A concentrate from brewers' yeast has much greater activity than acetate but the nature of the active principle is still obscure. From replacement studies with fatty acids and fat soluble substances, it is suggested that acetate may act as precursor of cell lipoids.

The folic acid group of factors.—Many outstanding problems in this field (2) have been greatly illuminated by the publication of the structure of several members of this complex. Analysis of the degradation products of "fermentation" *L. casei* factor led (34) to the synthesis of "pteroylglutamic acid"—N-[4-{[(2-amino-4-hydroxy-6-pteridyl) methyl] amino} benzoyl] glutamic acid—, which proved to have the biological activity of "liver *L. casei* factor" (i.e., active for both *L. casei* and *Strept. fecalis*). The pteroyl radical is defined as *p*-aminobenzoic acid linked via the amino group to the pterin residue. Vitamin B_c from liver and yeast is also identical with this substance (35). The existence of a factor (SLR), active for *Strept. fecalis* (*lactis*) *R* and inactive for *L. casei*, has been known for some time (36 to 38). It has now been shown (34) that pteroic acid (i.e., the molecule without glutamic acid) is active in the same sense, though it is not possible to decide whether the two substances are identical since relative activities are not stated. The fact that conversion of SLR factor to *L. casei* factor (LC) by washed cells of *Strept. fecalis* is greatly increased in the presence of fermentable carbohydrate (39) suggests that the change is an anabolic one. *Strept. fecalis* is presumably able to complete the synthesis of LC factor when given the less complex compound.

Other members of the folic acid group appear to be peptides of pteroylglutamic acid with glutamic acid. Thus vitamin B_c conjugate has been identified as pteroylheptaglutamic acid (35) and there is evidence that "fermentation" LC factor is a pteroyltriglutamic acid (34). The "fermentation" factor has considerable activity for *L. casei* but much less for *Strept. fecalis* (40, 41). It seems possible that the activity of this factor depends on the ability of the organism to hydrolyze it to free pteroylglutamic acid, and that *Strept. fecalis* is less active in this respect. A similar difference in the power of these two organisms to split a naturally occurring conjugate is indicated in another paper (42). An LC factor from yeast was reported earlier (43) as being less active for *Strept. fecalis* than for *L. casei*. If the factor contained some conjugate this observation might be explained along similar lines. Animal tissues are known to contain enzymes which hydrolyze conjugated folic acid; such enzymes are carboxypeptidases (35) and their kinetics have been studied (44).

It is doubtful, however, whether the compounds of known con-

stitution account for all the known activities of members of this group. Liver contains two chloroform soluble factors which can replace folic acid in the growth of *L. casei* and *Strept. fecalis* (45), and whose properties differentiate them from folic acid. Preliminary reports (46, 47) have been given of factors necessary for the growth of *L. casei* which are not identical with natural or synthetic folic acid; analysis of the effect of the natural factor on growth and acid production suggest that this material may be complex (47). Incubation with chicken liver increases the activity of LC factor as measured by *Strept. fecalis* assay (48); this may indicate that the factor preparations used contained conjugate, but other explanations are possible. The increase in this effect caused by pyracin has not been explained. Finally there is evidence (39) that LC factor, as synthesized from SLR factor by bacteria, is less stable than that from other sources.

The relationship of folic acid to sulfonamide action will be discussed later.

Metabolic Function and Metabolism of Growth Factors

The study of an essential growth factor is not completed by its chemical identification; it is then necessary to discover with what cell processes the factor is concerned and the precise mechanism of its action. Considerable attention has been given to this aspect of bacterial nutrition during recent years, and an attempt will be made to summarize the findings, and to indicate the types of methods which have been used in attacking this problem. Certain factors (e.g., thiamine, nicotinic acid, riboflavin) have been known for some time to be components of important enzyme systems, and this probably represents their principal metabolic function; the facts have been sufficiently covered by earlier reviews in this Journal [e.g. (1)]. Recent progress in this field has been made, in the main, by the following five methods, or by a combination of two or more of them:—

(*a*) Discovery that a component of an isolated enzyme system is identical with a known growth factor.

(*b*) Study of the metabolism of organisms grown on media deficient in the factor in question; followed by detailed investigation of any metabolic process which appears to

have been affected. This method, first used by Peters (49) in studies of vitamin B_1-deficient pigeon brain, has proved most valuable when applied to bacteria.

(*c*) Use of substances, often analogues of the factor (antimetabolites), which may specifically inhibit the utilization of the factor.

(*d*) Study of the metabolism of the factor itself and its linkage with other metabolic processes of the cell.

(*e*) The discovery, in growth experiments, that the factor may be replaced by a substance (X) of a different chemical type may suggest that the factor is involved in the synthesis of X or vice versa.

Information concerning the general type of reaction in which a factor is involved may sometimes be obtained indirectly. Thus it has been shown (50) with *Lactobacillus arabinosus* that growth in suboptimal concentration of biotin or pantothenate leads to the production of fewer organisms per unit of lactic acid produced than is the case with suboptimal nicotinic acid. It is therefore suggested that lack of biotin or pantothenate interferes to a greater extent with synthesis of protein than does lack of nicotinic acid, and that the latter is more concerned with glucose breakdown.

Vitamin B_6 group.—The function of pyridoxal phosphate as a component (coenzyme) of certain amino acid decarboxylase enzymes is clearly established and the evidence [obtained mainly by methods (*a*) and (*b*)] has been reviewed recently by Gale (51). Synthetic pyridoxal phosphate has been purified and evidence given that the phosphoryl radicle is attached to the phenolic group of pyridoxal (52). Further evidence that pyridoxal phosphate is the functionally active member of the B_6 group is provided by the finding (53) that pyridoxine, pyridoxal and pyridoxamine are converted to codecarboxylase by growing cultures of all organisms which can use them as a source of B_6; cell suspensions can also bring about this change in the case of pyridoxal and pyridoxamine, though with the latter the presence of a keto acid is also needed. In earlier work it was found (54) that nicotinic acid (as well as pyridoxine was necessary for tyrosine decarboxylase formation by *Strept. faecalis*. It has now been shown (55) that nicotinic acid has much less effect on the formation of the apoenzyme, and it is therefore suggested that it is involved, through the energy mechanisms of the cell, in the conversion of pyridoxal to the coenzyme

The intervention of pyridoxal phosphate in amino acid metabolism is not, however, limited to the decarboxylases. Snell (56) suggested that a pyridoxal⇄pyridoxamine transformation might play a catalytic role in transaminations, and (57) showed that reversible transamination between pyridoxal and glutamic acid occurred on autoclaving. Gunsalus and his colleagues (58) then found that synthetic pyridoxal phosphate functions as coenzyme with cell-free preparations of *Strept. fecalis* R catalyzing transamination between glutamic acid and oxaloacetic or pyruvic acid. The activating effect was observed both with apoenzyme from cells grown in pyridoxine-deficient medium, and with apoenzyme from resolution of the complete enzyme. It was next shown (59) that pyridoxamine phosphate is equally active in this system (though inactive as codecarboxylase), thus strengthening the view that the mechanism of cotransaminase action depends upon the intermediate transfer of amino groups between pyridoxal and pyridoxamine phosphates. Pyridoxal phosphate also appears to be active as coenzyme in some animal transaminase systems though not in all (60 to 64).

Working with cell-free preparations of *Neurospora*, Umbreit *et al.* (65) have found that synthetic pyridoxal phosphate also activates the enzyme system which brings about the direct condensation of indole and serine to form tryptophane (66). A wider role of this factor in amino acid synthesis is possibly indicated by the finding that the growth requirement of *L. arabinosus* for phenylalanine, tyrosine and arginine, and of *L. casei* for aspartic acid, can be replaced by pyridoxine plus carbon dioxide, but not by either alone (67).

It is clear that members of the vitamin B_6 group have a general function in amino acid metabolism involving decomposition, interconversion and synthesis.

Pantothenic acid.—Using method (*b*) with *Proteus morganii*, evidence has been obtained (68, 69) that pantothenate is involved in the aerobic and anaerobic metabolism of pyruvic acid and possibly other substrates (in particular C_4 and C_5 dicarboxylic acids, but not glucose); the oxidation of pyruvate to acetate has been especially implicated. McIlwain (70, 71) has attacked the problem by a combination of methods (*b*), (*c*), and (*d*), and by a quantitative examination of the effect of the factor and of a specific "antimetabolite" inhibitor (pantoyltaurine) on the rate and extent of

growth. Using hemolytic streptococci as main test organism he finds that pantothenic acid is itself metabolized provided that glycolysis is taking place simultaneously. Since pantothenate disappearance, but not glycolysis, is inhibited by pantoyltaurine; and since glycolysis by pantothenate-deficient cells is stimulated only slightly by pantothenate; it is concluded that this factor is not involved directly in the glycolytic mechanism. It appears, however, that the metabolism of pantothenate is intimately connected with its growth factor function, because (*a*) the rate of metabolism is similar with growing cultures and cell suspensions, and (*b*) there is correlation between the inhibitory effect of a number of pantothenate analogues on growth and on pantothenate disappearance. McIlwain suggests that the data as a whole are compatible with the view that the coupled metabolism of pantothenate and glucose yields an unknown substance (s) which is essential for growth, and that this reaction is inhibited by pantoyltaurine.

Two findings come under the heading of method (*e*). It has been shown (72) that the requirement of two strains of *Staph. aureus* for tryptophane can be met by pantothenate if glucose is also present (see p. 619). With *Cl. botulinum* growth occurs in a pantothenate-free medium provided that thiamine and choline are both present (73); whether this means that the latter are required for the synthesis of pantothenate or vice versa remains to be determined. It is also reported (74) that threonine synthesis by yeast is aided by pantothenate.

The metabolic role of this factor is not yet clear. It appears to be involved in several systems of rather different types, but more rigid analysis may show that its function is concerned with a key reaction common to these systems.

Glutamine.—McIlwain (75) has studied this factor by method (*d*). Glutamine is decomposed by growing cultures of hemolytic streptococci (which require it for growth), and also by cell suspensions when glucose fermentation is proceeding concurrently; the glutamine is hydrolyzed almost quantitatively to ammonia and glutamic acid. The rate of glucose metabolism is almost doubled when glutamine is present, but the products of fermentation are not changed. Although Gale's (76) observation that absorption of glutamine and glutamic acid by gram-positive cocci can only proceed concurrently with an energy yielding process might ac-

count for part of these findings, the stimulation of glycolysis by glutamine remains to be explained. McIlwain suggests that this metabolite plays a part in streptococcal glycolysis, by a mechanism possibly involving an ammonia transfer, and there is some evidence that the hydrolysis of glutamine is correlated with its growth promoting activity. The finding (77) that the sulfoxide of methionine may inhibit the synthesis of glutamine from glutamic acid may provide a useful specific inhibitor for future studies.

Biotin.—Winzler *et al.* (78) have shown an interrelationship between biotin and ammonia with *Saccharomyces cerevisiae* which may provide a clue as to the function of this factor. Cells grown in media containing suboptimal biotin showed greatly depressed rates of respiration and fermentation; these rates were increased by the addition of biotin plus ammonia but not by either alone. The assimilation of biotin by deficient cells was found to depend on the presence of glucose and phosphate, and that of ammonia in turn upon the presence of biotin. The suggestion has also been made (79) that biotin may function as a coenzyme of carbon dioxide transfer, since the molecule contains a ureylene structure which can be opened or closed by yeast with loss or gain of carbon dioxide.

Miscellaneous factors.—Evidence has been presented that (*a*) *m*-inositol is involved in fermentation by *Cl. saccharobutyricum* (80), (*b*) iron protoporphyrin in the reduction of nitrate to nitrite by *Hemophilus influenzae* (81), and (*c*) strepogenin in the metabolism of glutamic acid by *Strept. fecalis* and *L. casei* (82).

p-Aminobenzoic acid; mode of action of sulfonamides.—Since it has been argued by Henry (83) and Sevag (84) that *p*-aminobenzoic acid (*p*-AB) is not a growth factor within the usual meaning of the term, it is necessary to recapitulate the evidence. Twelve bacterial species, representing six genera, were listed in 1945 (85) as requiring, or being stimulated by, this substance; most of them did not grow appreciably without it, and the "blank," if any, was no higher than in studies with other growth factors on similar basal media. It has since been shown that *p*-AB is required by *Leuconostoc mesenteroides* Pd-60 (86) and *Rhodopseudomonas palustris* (28), and stimulates the growth of *Cl. botulinum* (30). Striking evidence that this substance is a true essential metabolite has come from studies of induced mutants. Treatment of several molds (87 to 89)

and *E. coli* (90, 91) with x-rays, U. V. light or mustard gas yields mutants specifically requiring *p*-AB for growth; mutants requiring well-established growth factors are obtained (92) under precisely the same conditions. It is also suggested (83, 84) that *p*-AB cannot be a growth factor since it often reduces growth at higher concentrations. A similar effect (at concentrations above the optimum) is shown by nicotinic acid with *Shigella* (93), thiamine and riboflavin with *Rhizobium* (94) and β-alanine with *Saccharomyces* (95). The reviewer is unable to see any justification for not considering *p*-AB as a normal growth factor. Many organisms which do not require preformed *p*-AB for growth have been shown to synthesize for themselves material with the same biological activity, and there are analogous findings for all other growth factors (85).

Since the growth inhibitory action of the majority of sulfonamides is annulled in a competitive manner by *p*-AB, it is likely that the elucidation of the metabolic function of this factor will illuminate the basic mode of action of these drugs. Furthermore the sulfonamides may be used as specific inhibitors in such studies. It was suggested (96), as a working hypothesis, that sulfonamides competitively inhibit an enzyme reaction involved in the utilization of *p*-AB. Considerable information as to the nature of this utilization is now to hand, and the interrelationships of *p*-AB with (*a*) the purine bases, (*b*) methionine and (*c*) folic acid are of particular interest.

The *p*-AB requirements of *Acetobacter suboxydans* (97) and some species of *Lactobacilli* (98, 99) are quantitatively reduced, and in the case of *Cl. acetobutylicum* (100) probably abolished, in the presence of purine bases. The concentration required is about a thousand fold that of *p*-AB, and rather better results are usually obtained with a mixture of bases, although adenine alone is almost as active. With *L. arabinosus* and *L. pentosus* adenine, guanine, xanthine and hypoxanthine reduce the amount of *p*-AB required to antagonize sulfonamide (99), while with *Eremothecium Ashbyii* and yeast, adenine itself has about one-tenth the anti-sulfathiazole activity of *p*-AB (101). There is also evidence (102) that yeast grown in the presence of sulfathiazole has a diminished nucleic acid content. Guanine and xanthine are sulfonamide antagonists (*E. coli*) but only in the presence of methionine (103). A number of other studies have implicated this amino acid. At high concentra-

tions it can replace the *p*-AB requirement of certain *Lactobacilli*, and has antisulfonamide activity with *L. casei* (99) and *E. coli* (103 to 105), though only over a limited range of drug concentration. Other amino acids have slight activity (103, 106) but only if methionine is present (103). Harris & Kohn (104) suggest that low concentrations of these drugs inhibit the synthesis of methionine, and that this synthesis, as well as others important to the cell, depends on the utilization of *p*-AB.

Shive & Roberts (107) find with *E. coli* that the critical ratio sulfonamide/*p*-AB is raised threefold if methionine is present and a further threefold if guanine or xanthine is also added. Similar experiments with *L. arabinosus* gave a tenfold increase in the presence of adenine, and it is deduced that purines and methionine are products of reactions involving *p*-AB. These interrelationships have been much clarified by recent work with an induced mutant of *E. coli* requiring *p*-AB. It is found (90) that this factor may be replaced by amino acid mixture (of which methionine is the most important constituent) plus adenine, guanine, xanthine and relatively high concentrations of thymine. Growth on this meduim is subcultivatable and highly resistant to sulfonamides; and it is concluded that, with *E. coli* at least, *p*-AB functions in the synthesis of the purines, thymine, and one (probably methionine) or more amino acids. It will be recalled that there is also evidence (2) that folic acid may be concerned with the synthesis of purines and thymine.

The pteroylglutamic acid molecule (see p. 608) contains a *p*-AB moiety, and this suggests at once that *p*-AB is required for the synthesis of this factor. The production of "folic acid" by mixed cultures from the fowl intestine was found earlier (108) to be stimulated by *p*-AB, while growth of *Mycobact. tuberculosis* on a medium containing *p*-AB led (109) to the production of material with the properties of vitamins B_{10} and B_{11} (members of the folic acid group).

If the only function of *p*-AB is to act as a precursor for folic acid synthesis it might be expected (*a*) that organisms requiring *p*-AB would grow if given an equivalent concentration of folic acid (providing the latter can be absorbed by such organisms and information on this point is lacking), and (*b*) that folic acid would have antisulfonamide activity of a noncompetitive type (since the product of the inhibited reaction is added). Synthetic pteroylglutamic

acid, however, is inactive as growth factor for the mutant *E. coli* requiring *p*-AB (90) and is less active than *p*-AB (never more than 4 per cent) for several other organisms (110); it is not however certain (see p. 608) that the synthetic substance is the only one of this chemical group required by bacteria. Inhibition by sulfonamides of the growth of *Strept. fecalis* (Ralston),[2] *L. arabinosus* and several enterococci is antagonized noncompetitively by pteroyglutamic acid at a concentration as low as that which promotes growth of organisms requiring this factor, and it is concluded that the synthesis of pteroylglutamic acid from *p*-AB is the point of sulfonamide inhibition with these organisms (111). This phenomenon is not, however, obtained with *E. coli*, *Staph. aureus* or *Diplococcus pneumoniae*. Noncompetitive antagonism is also found (110) with *Streptobacterium plantarum* 10 S at a pteroylglutamic acid level of the same order as that promoting growth (50 to 100 times that of *p*-AB). Interference by these drugs in the synthesis of folic acid is also suggested by observations (99, 111) that organisms requiring preformed SLR or LC factors are particularly resistant to sulfonamides, and (112) that the production of folic acid by growing cultures of *E. coli* is inhibited by sulfanilamide.

p-Aminobenzoylglutamic acid is reported to be 5 to 10 times more active than *p*-AB as sulfonamide-antagonist with *Streptobact. plantarum* 10 S (113). This is not the case with other organisms (114, 115) but the observation has now been repeated (110) with the 10 S strain and at several levels of sulfanilamide and sulfathiazole concentration; the relationship is, however, a competitive one, so it is presumably not the synthesis of this moiety of folic acid which the drugs inhibit.

There is thus strong evidence that *p*-AB is concerned in the synthesis of folic acid, purines, thymine and methionine, and that sulfonamides interfere with these functions. In the case of the nucleic acid derivatives, folic acid is possibly the primary intermediate, but there is as yet no hint as to the mechanism of the effect of *p*-AB on methionine anabolism. This review is of necessity restricted to the relationship between the metabolic function of *p*-AB and the action of sulfonamides, but if these drugs do interfere (as seems probable) with the synthesis of such key substances

[2] This organism is not *Strept. fecalis* R.

to the cell as the purines and pyrimidines, it is reasonable that many other cell processes should be secondarily affected. Thus the effect of these drugs on respiration (83, 84) may arise from the fact that adenine is involved in the structure of the respiratory coenzymes. Two groups of workers (115, 116) have suggested that sulfonamides may inhibit synthetic reactions leading to proteins and peptides; such processes may well involve both nucleic acid and methionine.

Problems of Biosynthesis

It is generally accepted that the substances recognized as growth factors for particular microorganisms are essential for the growth of most microorganisms, but that some organisms can synthesize a given factor whilst others cannot (19, 117). In a number of organisms requiring the same factor, the power to synthesize may have failed at different stages of the series of reactions by which the synthesis is accomplished, and the provision of intermediates above the point of failure will permit the synthesis to be concluded and the organism to grow. Thus organisms are known which require substances of increasing complexity (ammonia, nicotinic acid, nicotinamide, nicotinamideriboside) for the synthesis of the phosphopyridinenucleotide coenzymes [for references see (137)]. Similar evidence as to the path of synthesis can often be obtained with a single organism if a number of mutants, each requiring ultimately the same essential metabolite, are available for study (92). Here again the mutants may fail at different stages of the synthetic process. This approach is made more practicable if the slow natural mutation rate can be increased, and the outstanding work of Beadle and co-workers (92) with x-ray or U. V. induced mutants of *Neurospora* has proved the value of the technique. A recent example (118) concerns the biosynthesis of thiamine. It seems only a matter of time before equally fruitful results are obtained with bacteria. Biochemical mutants needing a variety of essential metabolites (including amino acids) have been obtained by the action of x-rays and U. V. light (91, 119 to 121) or bacteriophage (122) on *E. coli*, and such mutants appear to be stable (121). The induction of mutants in *Neurospora* by mustard gas (123) may also prove applicable to bacteria. A rapid method of

separating such mutants from normal organisms has been described (91).

When an exacting organism is grown in the presence of the required growth factor it sometimes happens that the intermediate compound corresponding to the point of failure of the synthetic mechanism accumulates in the system. A nicotinamide-requiring mutant of *Neurospora* (124), growing in the presence of this factor, produces a substance which promotes the full growth of a similar, but genetically distinct, mutant. The substance is presumably a precursor in the synthesis of nicotinamide and there is evidence that it is an oxypyridinecarboxylic acid. Accumulation of precursor may also occur (125) when growth is limited by specific inhibitors of the utilization of a metabolite. Such growth inhibitions (e.g., by metabolite analogues) may, if competitive in nature, yield further information; the addition of the product of the inhibited reaction will promote growth irrespective of inhibitor concentration (126), or, if the inhibitor affects also other reactions, a higher concentration may be required to suppress growth (127).

Synthesis and breakdown of tryptophane.—The case of tryptophane illustrates well the general methods outlined above. The finding that the trytophane requirement of *Bact. typhosum* could be met by indole (128), and of certain species of *Lactobacilli* by indole or anthranilic acid (129), suggested that these two substances are intermediates in the synthesis. Studies with mutants of *Neurospora* (66) confirmed this idea and led also to the discovery that, with this organism, the final stage in the synthesis is a direct condensation of indole and serine; pyridoxal phosphate has now been shown (65) to be involved in the condensation mechanism. Tatum & Bonner (66) also found some evidence of tryptophane formation when *E. coli* was incubated with indole and serine. Fildes (125) was unable to confirm this (possibly owing to strain difference—the organism also breaks down tryptophane to indole, so the balance may be delicate), but obtained evidence by other methods for the role of indole and serine as intermediates with bacteria. Using a strain of *Bact. typhosum* trained to grow without either indole or tryptophane, and indoleacrylic acid as a specific inhibitor of tryptophane synthesis from indole (126), he found an accumulation of indole when growth was partially suppressed by the inhibitor; furthermore growth inhibition was much reduced if

serine were added. Schweigert *et al.* (130), using an amino acid medium not containing serine, found that indole has only a quarter of the growth-promoting activity of tryptophane for *L. arabinosus*, but that this is doubled if serine is also added. Since the increased activity still falls short of that of tryptophane, it is thought that another substance (s) (shown to be present in hydrolyzed casein) is also involved. The action of pantothenate (plus glucose) in replacing the trytophane requirement of two strains of *Staph. aureus* (72) may reflect a function of this factor at an earlier step in the synthesis.

The mechanism of the degradation of trytophane to indole has been an outstanding problem for many years. In recent work (131), the suggestion that *β-o*-aminophenylacetaldehyde is an intermediate (132) has been criticized on the grounds that dimedon inhibits spontaneous indole formation from this substance, but not from tryptophane by bacteria. Tatum & Bonner (66) thought that breakdown might occur by a direct split to indole and serine (i.e., the synthetic reaction in reverse) and found indole production by *E. coli* to be slowed down by serine. Baker *et al.* (131) have extended their earlier work (133) on essential structural conditions for the tryptophane-indole reaction and find support for their suggestion that the alanine side chain is removed as a whole. Strong direct evidence for this mechanism has come recently from the same laboratory (134, 135): in the presence of mepacrine, washed cells of *E. coli* produce initially some indole without concurrent oxygen uptake, and during this period alanine (but not serine) can be detected by partition chromatography.

Other essential metabolites.—A number of papers presenting evidence for possible intermediates in the synthesis of several growth factors were reviewed last year by Snell (2); such evidence was based mainly on the capacity of substances to promote growth in the absence of the factor in question. Specific "antimetabolite" growth inhibitors are being used increasingly in this connection. Inhibition of growth of *E. coli* by β-hydroxyphenylalanine and thienylalanine (136) is counteracted by phenylalanine and also, though less effectively by tryptophane, and it is suggested that the latter is involved in the synthesis of phenylalanine; in the case of thienylalanine, however, other workers (137) find tryptophane as effective as phenylalanine. Growth inhibition (*E. coli*) by *p*-hy-

droxyaspartic acid is completely annulled by aspartic acid, while pantothenic acid and β-alanine raise the concentration of inhibitor required to suppress growth (127). It is concluded therefore that this inhibitor prevents at least two essential functions of aspartic acid, one of which is to act as precursor (by decarboxylation) of the β-alanine moiety of pantothenic acid; the nature of the other function (inhibited at higher concentrations) is not known. The finding (138) that pantothenic acid antagonizes the growth inhibitory effect of salicylate, and that pantoate is more active in this respect than pantolactone, indicates that pantoate rather than the lactone is the precursor of pantothenate: this view is supported by other work on the relative activities of these substances in replacing pantothenate as a growth factor (139, 140).

Certain growth factors are known to be components of enzyme systems and recent work with "antimetabolites" may throw light on the mechanism of formation of the ultimate complete enzyme. The utilization of diphosphothiamine for growth of *L. fermentum* and *Pencillium digitatum* is more susceptible to inhibition by pyrithiamine than is that of free thiamine, and these results (141) are interpreted as indicating that thiamine is attached to the apoenzyme before phosphorylation. The effect of the same inhibitor on the growth of thiamineless mutants of *Neurospora* is overcome more effectively by pyrimidine plus thiazole than by thiamine itself (118). It is considered possible therefore that linkage of the moieties may occur only after union with the enzyme protein. In the case of riboflavin, analogous findings with *L. casei* using lumiflavin as inhibitor suggest its combination with apoenzyme before conversion to riboflavin phosphate or flavin-adenine-dinucleotide (142).

The fact that both boron and *m*-inositol (though not succinic acid) reverse the inhibitory effect of malonate on fermentation by *Cl. saccharobutyricum* may indicate that boron is a coenzyme in the synthesis of inositol (80).

Control of synthetic mechanisms.—McCarty (143) has contributed an excellent review on the transformation of pneumococcal types. The identification of the substance controlling the synthesis of the polysaccharide of Type III *Pneumococcus* as a desoxyribonucleic acid has been made more rigid by work (144) with a highly purified desoxyribonuclease. The value of this enzyme as a specific

reagent is increased by the fact that its activation by magnesium is inhibited by citrate while that by manganese is not. By using citrate to inhibit the desoxyribonuclease liberated from pneumococci themselves on lysis, McCarty & Avery (144) were able to improve the yield of extracted Type III transforming factor fivefold, and have isolated similar factors from Type II and VI organisms. These were again desoxyribonucleic acids. It is pointed out (144) that only a small proportion of the molecules of a preparation are active as transforming principle since rough (R) strains give a similar desoxyribonucleic acid fraction but without activity in this sense. This work must surely be a classic in the technique of elucidation of structure of large molecules by enzymic analysis. Boivin *et al.* (145) have shown the presence in *E. coli* of a similar factor controlling the synthesis of polysaccharide, and able to bring about transformation of Types via the rough form. There is evidence that the factor is a polymerized thymonucleic acid and that transformation involves also loss of ability to ferment sucrose.

Miscellaneous.—The phenomenon of oxidative assimilation has been fully covered by Clifton in a recent review (146). Such assimilation involves the synthesis of substances of higher energy content, and Clifton favors the concept that such changes may occur by the intimate coupling of anabolic and catabolic reactions without energy transfer from a materially independent system. The synthesis of polysaccharides from sucrose by cell free enzyme preparations of bacteria was dealt with last year (3); in a recent paper (147) the kinetics of a more potent preparation from *Leuconostoc mesenteroides* are described.

Factors Associated with the Toxicity and Virulence of Bacteria

Isolation and chemical nature of bacterial toxins.—The crystallization of the toxin of *Cl. botulinum* Type A has been achieved by two groups of workers in the same laboratory using different methods. The procedure of Lamanna *et al.* (148, 149) involves shaking with chloroform as an important step: this removes inactive protein and shifts the critical pH of acid precipitation of the toxin; the salt concentration during purification is also an important factor in increasing the stability of the toxin. Abrams *et al.* (150) use sodium sulfate and acid precipitations. In both cases the

crystalline toxin has the properties of a protein of the globulin type. The toxicity remains unchanged on recrystallization and there is further evidence of the homogeneity of the protein from sedimentation, electrophoresis and absorption spectrum studies (148, 150 to 152). The mol. wt. is computed to be $9\times10^5-1.1\times10^6$ (151, 152) and a preliminary analysis by microbiological assay methods indicates nothing unusual in the amino acid composition of the protein (153). The crystalline toxin is the most poisonous substance known, the toxicity for mice being *ca.* 2×10^8 LD_{50}/mg. nitrogen, or 32×10^9 LD_{50}/gm. dry toxin; on the basis of a mol. wt. of 9×10^5 it is calculated (151) that a single mouse LD_{50} is equivalent to 2.1×10^7 molecules.

Good growth and toxin production by *Cl. botulinum* Types A and B on media of known composition have also been reported (30). With Type A the titer of toxin was one-tenth to one-twentieth of that obtained on the complex medium used for the isolation of the crystalline toxin.

A second toxin obtained in crystalline form is that of *Cl. tetani*. Pillemer *et al.* (154) using a method (155) in which methanol, under rigidly controlled conditions, is used to precipitate in turn active and inactive materials, finally isolated a crystalline toxin of potency 5 to 7.5×10^7 mouse M.L.D./mg.nitrogen. The parent high titer toxic culture filtrate was obtained with a poorly sporing variant of *Cl. tetani* (156) on a complex medium (157). The product has constant biological activity on recrystallization and has the general properties of a protein, but no further data concerning its homogeneity have yet been published.

The isolation of these two toxins in crystalline form and a high state of purity will greatly facilitate future investigations concerning their mode of action. The extremely high order of biological activity coupled with their protein nature is suggestive of the possibility that they may be enzymes.

There have also been many reports (too numerous to give in detail here) of progress with other toxins and related factors. Such progress has been mainly in regard to suitable media and conditions for high titer toxin production, and with partial purification of the active principle. Although concentrates have often shown protein properties, it would be premature to draw conclusions as to their chemical nature. There appear to be no special factors for

toxin production (as opposed to growth) when staphylococcal enterotoxin is produced on a chemically defined medium (157a).

Enzymic nature of certain toxins of Cl. welchii.—It is now five years since Macfarlane & Knight (158) demonstrated the presence of a lecithinase enzyme in preparations of the α-toxin of Type A *Cl. welchii* and obtained strong evidence for the identity of this enzyme with the actual toxin. In the course of that work it was found that protein-free extract of horse muscle contains a factor which stimulates toxin (and enzyme) production. Rogers & Knight (159) have now purified this factor and find the properties of active fractions suggestive of an amino sugar; glucosamine and *N*-acetylglucosamine proved to have activity equivalent to 30 per cent of that of the factor. The iron concentration of the medium became critical as the factor was purified. A general relationship between *in vitro* α-toxin or lecithinase production and virulence of the organism is brought out by recent distribution studies (160, 161).

Following up an observation (162) that incubation with *Cl. welchii* Type A filtrates causes rapid disintegration of muscle, and that this is due (163) to destruction of the reticulin scaffolding, Oakley *et al.* (164) have confirmed the presence of a collagenase enzyme in such filtrates and worked out an ingenious method of assay. Since the enzyme is immunologically distinct from other recognized components of the toxin complex, and may be responsible for the pulping of muscle in human gas gangrene, the authors have christened the enzyme, "κ-toxin."

Hyaluronidase (*spreading factor, invasin*).—The presence of the mucopolysaccharide, hyaluronic acid, in skin, and of the enzyme, hyaluronidase, in pathogenic bacteria, has led to the concept (165, 166) that hyaluronidase plays an important role in bacterial invasion by depolymerizing the mucoid ground substance of connective tissue. If this is the case it seems that invasive power is not a controlling factor in determining overall virulence of an organism, since recent studies with many strains of staphylococci (167, 168) and *Cl. welchii* (160, 161) have failed to show a correlation between virulence and hyaluronidase production. Haas (166) has investigated the defence mechanism of the host against invasin (hyaluronidase) and has shown the presence in normal blood plasma of an enzyme (anti-invasin I) which destroys in-

vasin; further enzymes in bacteria (proinvasin I), and in plasma (anti-invasin II), destroy anti-invasin I and proinvasin I respectively, and it is suggested that the quantitative balance of these enzymes in the host-bacterial system determines whether or not invasion will occur. Several detailed studies of the kinetics and other properties of hyaluronidase have been made (169 to 171). Enzyme production on a simplified medium has been obtained, though some organisms require complex factors; and the enzyme is adaptive with some organisms but not with others. Although enzymes from various sources all hydrolyze the substrate with liberation of reducing sugars and acetylhexosamine, there is considerable variation, according to the source, in the degree of hydrolysis and molecular size of the product, and this is reflected in the differing abilities of such products to promote adaptive formation of the enzyme: it seems possible that hyaluronidase is a complex of enzymes. Activity against other substrates (e.g., chrondroitin sulphuric acid) also varies with the source of the enzyme.

Bacterial pyrogen.—Pyrogens from several organisms growing on synthetic media have been concentrated to an activity of 0.01 μg./kg. (rabbit). The chemical properties of the purified materials, which are protein-free, suggest that they may be aminopolysaccharide in nature (172).

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NITROGEN METABOLISM

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Proteins form an essential structural and functional component of the living cell and an understanding of the chemical processes involved in the formation and breakdown of protein is essential to the ultimate understanding of cellular growth and of chemical interference with growth. Microorganisms are equipped with a wide variety of enzymes which are able to attack proteins and their derivatives and, in turn, bring about a reorganization of the raw material so provided into microbial protein. Until recent times studies of the nitrogen metabolism of microorganisms had been confined in the main to the investigation of the breakdown of proteins, peptides, amino acids, etc., but the introduction of new techniques has led during the last few years to a shift of emphasis to the study of anabolic changes. The proteins of microorganisms appear to be essentially similar in their composition to those of other living cells but microorganisms are outstanding in their abilities to synthesize or assimilate amino acids and to collect and concentrate these in the right proportions for rapid synthesis of protein which usually appears in the form of new cell-material. We can already distinguish well-defined stages in the anabolism of protein and this review will be concerned mainly with a summary of our knowledge of these stages with special reference to the main problem of growth.

If we take ammonia as the simplest starting material for the synthesis of cellular protein (omitting the nitrogen-fixing organisms for the present) we can recognize the following stages: assimilation of ammonia, synthesis of amino acids, assimilation of amino acids, interchange of amino acids, synthesis of peptides, and condensation into protein. On the other hand we have degradation of exogenous protein through similar stages to ammonia but, as will be seen, it does not follow that the degradation of protein is necessarily accomplished by reversal of the similar stages involved in protein synthesis. These stages will now be considered in detail.

Synthesis of Protein

Assimilation of ammonia.—It is common knowledge that many bacteria, yeasts and fungi can grow in a medium containing ammonia as sole source of nitrogen; these organisms must therefore be able to assimilate ammonia but there are few studies, up to the present, of the conditions which control such assimilation. Winzler, Burk & du Vigneaud (1) have, however, shown that the assimilation of ammonia by *Saccharomyces cerevisiae* is dependent upon the biotin content of the cells. The assimilation of ammonia by normal biotin-rich cells is also dependent upon glycolysis, requiring the presence of both glucose and phosphate and being inhibited by sodium azide. If however the cells are grown in a biotin-deficient medium, the cells contain approximately one hundredth their normal biotin content and their rates of respiration and fermentation are approximately one twentieth those of normal cells. Such cells are unable to assimilate ammonia unless biotin is added to the external medium; when such addition is made, biotin is taken up by the deficient cells if glucose is present and ammonia is taken up if both biotin and glucose are present. The assimilation of biotin is independent of ammonia but that of ammonia is dependent on the presence of biotin. Following the addition of both biotin and ammonia to the deficient yeast cells, the rates of respiration and fermentation steadily increase but do not reach the normal value for about two hours after the addition of biotin. This increase of fermentation rate is dependent upon the addition of both biotin and ammonia; if ammonia is added two hours before the biotin, then the increase in fermentation rate takes place normally after the second addition; if the biotin is added two hours before the ammonia, then no effect can be seen on fermentation until after the second addition when the fermentation rate shows a small immediate increase followed by the usual steady increase over a period of one to two hours. These investigations indicate that biotin is intimately involved in the uptake of ammonia by fermenting cells and that the biotin-stimulated ammonia uptake produces a gradual rise in the rate of glucose fermentation. Winzler *et al.* suggest that these results indicate that biotin is involved in a synthesis of nitrogenous material connected with the metabolism of glucose. A somewhat similar effect may be involved in *S. haemolyticus*. McIlwain (2, 3) has demonstrated that the addition of glutamine to a washed suspension of this organism results in an increased rate of

glucose fermentation and glutamine is broken down inside the streptococcal cell to yield ammonia.

Burris & Wilson (4) have investigated the uptake of ammonia by *Azotobacter vinelandii* and find that within three minutes after exposure of the cells to ammonium sulphate containing N^{15}, the isotopic-N can be found in the amino acids of the organism. Of the amino acids investigated glutamic acid contained the highest N^{15} content. The distribution of N^{15} in the amino acids of the organism after this treatment was essentially similar to that found by Burris (5) after exposure of the cells to molecular nitrogen containing N^{15}, and this fact lends support to the ammonia theory of nitrogen fixation (4).

Synthesis of individual amino acids.—Of the many enzymes discovered which will deaminate amino acids, only two have proved to catalyze easily reversible reactions capable of supporting synthesis of specific amino acids in bacteria. These are the aspartase of Quastel & Woolf (6) and Virtanen & Tarnanen (7) and the glutamic acid dehydrogenase of Adler *et al.* (8), both enzymes being found in *E. coli* and related organisms. It seems that direct studies of enzymes by established techniques will not enable us to elucidate the synthesis of many of the common amino acids and the recent remarkable development of this branch of the subject has been initiated by the use of two indirect methods of study.

The first type of investigation was initiated by the studies of Gladstone (9) on the amino acid antagonisms that arise in the nutrition of *Bacillus anthracis*. He found that the addition of either valine, leucine or isoleucine to a mixture of amino acids, which otherwise formed a satisfactory nutrient medium, would inhibit growth. The addition of all three amino acids together had no inhibitory effect. Valine added alone is inhibitory and this inhibition is removed by the further addition of leucine; isoleucine alone is inhibitory and in this case the inhibition is removed only by the addition of both leucine and valine. A similar antagonism was demonstrated between serine and threonine and many similar examples of mutual antagonism have since been shown in other microorganisms (10 to 18). Gladstone suggested that where such an antagonism occurs between two amino acids they may arise from a single precursor in the course of biogenesis and that the presence of one blocks the synthesis of the other which then becomes essential. Growth is then restored by the further addition of

the amino acid whose synthesis has been blocked. It is not a large jump from blocking the synthesis of an amino acid by a naturally occurring amino acid to blocking it by a synthetic analogue. Harris & Kohn (14) observed that the growth of *E. coli* on a synthetic medium can be inhibited by ethionine and that this inhibition is completely reversed by methionine. Roblin *et al.* (19) found that methoxinine is inhibitory for both *E. coli* and *Staph. aureus* and that the inhibition is reversed by *l*-methionine. Du Vigneaud and his co-workers (20) showed that thienylalanine inhibits the growth of a strain of *S. cerevisiae* and that the inhibition is reversed by phenylalanine. A recent addition to this series of compounds is methionine sulphoxide which inhibits the growth of *L. arabinosus* and is competitively reversed by glutamic acid but has no inhibitory action in the presence of glutamine (21). It is suggested that the sulphoxide inhibits the amidation of glutamic acid.

Metabolic analogues of this nature can provide useful information on synthetic processes if their use leads to the accumulation of the substance whose further metabolism is inhibited. Fildes in a series of studies (22, 23, 24) has utilized this fact to elucidate the stages in the synthesis of tryptophane by *Eberthella typhosa* and *E. coli*. *Eberthella typhosa* requires tryptophane for growth although it can in some cases be trained to synthesize this amino acid (25). The tryptophane-exacting strains can utilize indole: it follows that tryptophane is synthesized from indole and that the synthetic inability lies in the formation of the indole nucleus. Indoleacrylic acid inhibits the growth of *E. coli* and *Eberthella typhosa* but the inhibition is noncompetitively reversed by tryptophane, a trace of tryptophane being sufficient to permit growth. Indoleacrylic acid must block some final stage in the synthesis of tryptophane and since its action is abolished by tryptophane but not by indole, it follows that this stage lies somewhere between the formation of indole and the synthesis of tryptophane therefrom. If this is true the nonexacting strain of *Eberthella typhosa* should synthesize indole in the presence of indoleacrylic acid. When subtoxic amounts of the inhibitor are added to growing cultures indole can be shown to accumulate in quantities greatly in excess of those which might arise from the indoleacrylate itself.

The synthesis of tryptophane has also been investigated by the other new method of approach, the study of nutritionally exacting mutants. Snell (26) has shown that the tryptophane requirement of

L. arabinosus and *L. casei* can be replaced by indole or anthranilic acid and further studies (27) indicated that anthranilic acid is the precursor of indole in the biogenesis of tryptophane. This approach could be extended if it were possible to investigate the nutritional requirements of mutants of the same organism in which the synthetic inability occurred at different steps in the same anabolic process. Such an extension has been made possible by the investigations of Tatum, Beadle and their co-workers on x-ray induced mutants of *Neurospora* and, later, of *E. coli*. The wild *Neurospora crassa* mould grows and synthesizes all its requirements in a simple medium consisting of salts, a suitable source of carbon, and biotin. If the mould is irradiated with x-rays its rate of mutation is greatly increased and a series of mutants are produced which differ both biochemically and genetically from the wild strain. Genetical studies indicate that each mutant differs from the parent by the alteration of one gene while biochemical studies indicate that the alteration of the gene is accompanied by the loss of one enzyme. If an essential metabolite is synthesized in a series of steps each catalysed by a separate specific enzyme, it should be possible in theory to produce a series of different mutants each of which would lack one of the enzymes in that synthetic chain. A study of the nutritional requirements of the members of the series should then serve to elucidate the components of the synthetic chain (28). Thus two genetically different mutants of *Neurospora* have been found both of which are exacting towards tryptophane (29) and both of which can utilize indole while only one can utilize anthranilic acid. It follows that anthranilic acid is a precursor of indole in tryptophane synthesis. Tatum & Bonner (30) studied the indole-using mutant and showed that indole disappears from the medium during growth and that the rate of disappearance is accelerated by and a function of the concentration of *l*-serine. The *Neurospora* mycelium grown in a tryptophane-deficient, indole-containing medium is found to contain tryptophane and this amino acid can also be found in the medium after growth, the medium tryptophane being equivalent to 30 to 50 per cent of the indole disappearing (31). The presence of *l*-serine is essential for the tryptophane synthesis so these workers conclude that the final reaction in tryptophane synthesis is a direct condensation of indole and *l*-serine. Umbreit, Wood & Gunsalus (32) have now demonstrated the synthesis in the presence of a cell-free homogenate of *Neurospora*

sitophila and find that the activity of the preparation decreases steadily but can be restored by the addition of pyridoxal phosphate which is presumably the prosthetic group of the enzyme concerned.

Further studies with x-ray induced mutants have shown that the synthesis of arginine in *Neurospora* proceeds through ornithine and citrulline and that there are at least four steps involved in the synthesis of ornithine. Since the organism also possesses arginase and urease, a typical "Krebs ornithine cycle" takes place in this organism (33). A further mutant has been found which is exacting not to one amino acid but to a mixture of valine and isoleucine in certain proportions (17). It appears that this strain is genetically blocked in the conversion of α-keto-β-methyl-*n*-valeric acid to isoleucine. The keto acid therefore accumulates and this, in turn, inhibits the synthesis of valine from α-keto isovaleric acid. Consequently a single genetic block in this case gives rise to a double nutritional requirement. The mutant cannot use "keto isoleucine" but can use "ketovaline" in the presence of isoleucine. A lysine-exacting mutant has been discovered (34) but no evidence has yet been published concerning the possible precursors of lysine. The growth of this mutant is inhibited by arginine if the ratio of arginine to lysine in the medium approaches the critical value of 2:1 although arginine has no inhibitory effect on the wild strain. The major advances in this field have so far been made with mutants derived from *Neurospora* but Tatum (35) has now produced amino acid exacting mutants of *E. coli* and we can expect that similar studies will shortly be possible with such organisms.

Assimilation of amino acids.—It has long been known that some organisms are able to synthesize all their nitrogenous material from inorganic sources while others have a synthetic disability towards certain amino acids. Among the *Eubacteriales* the organisms which can utilize ammonia as a sole nitrogen source are, generally speaking, gram-negative while many gram-positive species require a wide variety of amino acids for optimal growth. During the last three years studies have appeared of the amino acid requirements of *L. casei* (36), *L. arabinosus* (37, 38), *Strep. faecalis* (39, 40), *Strep. lactis* (41), *Mycobacteria* (42), *Acetobacter suboxydans* (43), *N. intracellularis* (44), *B. anthracis* (45), *Pr. morganii* (46), and *Leuconostoc mesenteriodes* (47).

Gale (48) has shown that gram-positive bacteria possess the capacity to assimilate certain amino acids and to concentrate them

in a free state within the internal environment. Lysine passes across the cell-wall by a process of free diffusion under an electrostatic gradient and the amount of free lysine held within the cell is probably dependent upon the negative charge in the cell. Glutamic acid and histidine however cannot diffuse through the cell-wall but require energy for their migration and this energy can be obtained from exergonic metabolism, such as glycolysis. In the cases of lysine and glutamic acid an equilibrium condition is set up between the internal and external concentrations such that the former is markedly greater than the latter. The maintenance of this concentration gradient across the cell-wall is dependent upon the intact nature of the cell-wall since it is destroyed by any interference with the structure of this wall whether this is by the action of heat, friction, or detergents (49). Taylor (50) investigated the capacity of a wide variety of bacteria to effect this concentration of free amino acids within the internal environment and found that it is confined to gram-positive species. *Staph. aureus* strains effect the highest internal concentration of glutamic acid among the organisms tested and no free amino acids could be detected in the internal environment of common gram-negative bacteria under any conditions tested. It seems probable that this property of assimilation of free amino acids represents the first step in protein synthesis by gram-positive bacteria, the concentration of the free amino acids within the internal environment compensating for the lack of synthetic ability towards such amino acies and for the low concentration and unsuitable proportion of such amino acids that may exist in external environment.

Within the cells of *S. faecalis* and *Staph. aureus* the concentrated glutamic acid acts as a source of this amino acid for condensation into peptide and protein (51) and also as a source of some other metabolic breakdown product whose nature has not yet been elucidated (52). The condensation of internal glutamic acid into protein structures occurs in growing cells only but the other form of metabolism occurs in both growing and resting cells. The level of free glutamic acid which is measured within the cell under various conditions thus represents a balance between the rate at which the amino acid is assimilated from the external environment and the rate at which it is metabolized within the cell; consequently any substance interfering with assimilation will lead to a falling internal concentration while any substance inhibiting the internal

metabolism will give rise to an increase in the free glutamic acid concentration. By detailed studies of this nature, the Cambridge school has been able to demonstrate that penicillin blocks the assimilation of glutamic acid by gram-positive bacteria (53), that triphenylmethane dyes penetrate the cell-wall and inhibit the internal metabolism of glutamic acid (52), and that sulphathiazole interferes, possibly in an indirect manner, with protein synthesis (51). The greater sensitivity of the gram-positive than of the gram-negative species to these antibacterial agents is probably explained by the greater dependence of the former on the capacity to assimilate and to concentrate free ready-formed amino acids rather than on the capacity to synthesize these substances as and when needed.

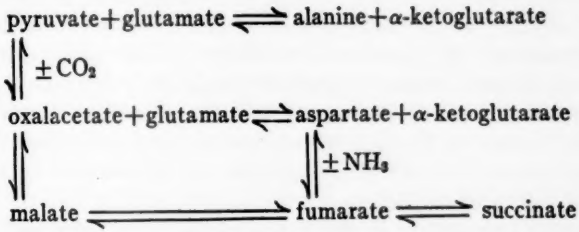
Interchange of amino acids.—The early work of Braunshteĭn & Kritsman (54) suggested that tissues should be able to synthesize a range of amino acids from the corresponding keto acids if one of the dicarboxylic amino acids was available to donate the amino group. Until recently it has not been possible to demonstrate the existence of a transaminase in bacteria since the many side-reactions involving the products masked the presence of the primary reaction. Lichstein *et al.* (55, 56) have now shown that many bacteria can carry out the reactions:

glutamic acid + oxalacetic acid $\rightleftharpoons$ α-ketoglutaric acid + aspartic acid

glutamic acid + pyruvic acid $\rightleftharpoons$ α-ketoglutaric acid + alanine.

Lichstein *et al.* (56) have obtained the cell-free transaminase from *S. faecalis* and demonstrated that it requires pyridoxal phosphate as a prosthetic group. Bacteria have thus been brought into line with other tissues with regard to their transaminase enzymes (57) and the prosthetic groups of these enzymes (58). It was originally suggested that transaminase systems would transfer the amino group from glutamic acid or aspartic acid to the α-position in any keto acid and so produce the corresponding natural amino acid but this suggestion has not been borne out in practice. Two transaminase enzymes which catalyze the two above-mentioned reactions but do not perform a more general transamination have been isolated from a number of tissues (57). The question therefore arises whether these enzymes are concerned in the interchange of amino acids as a process in protein synthesis or whether they form part of some respiratory mechanism involving the four and five-

carbon dicarboxylic acids and pyruvic acid. Many bacteria possess enzymes which are capable of synthesizing aspartic and glutamic acids (see above) so the only "new" amino acid synthesized by the transaminase system is alanine. In bacteria such as *E. coli* the existence has now been shown of all the enzymes required for the following series of equilibria:



but the full implication of this system in the bacterial economy is not yet obvious. We can see that there is a link between glycolysis and amino acid synthesis in that the formation of both alanine and aspartic acid depend upon the production of pyruvic acid. Carbon dioxide is also essential for the formation of oxalacetic acid and it may be that one of the essential roles of carbon dioxide in bacterial nutrition is linked with amino acid synthesis in this way. In this connection it is interesting to see that Lyman *et al.* (59) find that phenylalanine, tyrosine, and arginine can be replaced in the nutrition of *L. arabinosus* and that aspartic acid can be replaced in the nutrition of *S. faecalis* R, by pyridoxine in the presence of carbon dioxide but not otherwise. In *E. coli* it would appear that aspartic acid can be synthesized in two ways—from fumaric by the reversal of aspartase, or from glutamic by transaminase. Shive & Macow (60) have produced evidence that the precursor of aspartic acid during the growth of *E. coli* is glutamic acid since *dl-para*-hydroxy-aspartic acid inhibits the growth of this organism and this inhibition can be overcome either by a definite ratio of aspartic acid or by a wide range of concentrations of glutamic acid.

It is possible that the transaminase systems studied and the enzymes isolated in a cell-free state represent a portion of a more general system involved in the synthesis of amino acids. Evidence that some sort of amino acid interchange must take place is given by the work of Lyman *et al.* (59) mentioned above and of Stokes & Gunness (61) who find that *L. delbrueckii*, *L. casei*, and *L. arabino-*

sus require lysine, threonine and alanine if grown in a medium containing pyridoxin but these three amino acids are no longer essential for growth if the active derivatives pyridoxal or pyridoxamine are added. The role of pyridoxal and pyridoxamine as amino group carriers was first suggested in model experiments by Snell (62) who showed that pyridoxal can react with an amino acid to give the corresponding keto acid while being itself converted to pyridoxamine, which can in turn react with a keto acid to form the corresponding amino acid and liberate pyridoxal. These reactions were realized in biological form by Bellamy *et al.* (63) who showed that pyridoxamine will not act as codecarboxylase, but if incubated in the presence of *S. faecalis* and pyruvic acid, it is then transformed into an active codecarboxylase which is known to be replaceable by pyridoxal.

Peptide and protein formation.—Until about a year ago there was a complete blank in our knowledge of protein synthesis concerning the steps which intervene between the synthesis or accumulation of the constituent amino acids and their final appearance as protoplasmic protein. Then Angier *et al.* (64) announced the synthesis of the *L. casei* and *S. faecalis* factors and showed that both consisted of a pterine nucleus linked through *p*-aminobenzoic acid to one or two glutamic acid molecules. This recalled the earlier finding of Blanchard (65) that the *p*-aminobenzoic acid of yeast exists partially in a peptide form and Ratner, Blanchard & Green (66) have now isolated a peptide from yeast which consists of one molecule of *p*-aminobenzoic acid condensed with ten or eleven glutamic acid molecules and one molecule of an unidentified amino acid. It appears that glutamic acid has the property of condensing to form homoglutamic peptide chains—a property which is shown in the case of the unnatural isomer by the *d*-homoglutamic acid nature of the capsular polypeptide of *B. anthracis* and *B. mesentericus* (67, 68).

Since the discovery by Woods (69) of the antisulphonamide action of *p*-aminobenzoic acid, a search has been carried on for the metabolic function of this substance and it seems that we may now be getting some light on this problem. Lampen & Jones (70) investigated the antisulphadiazine action of *p*-aminobenzoic acid and related compounds using *Strep. faecalis* as test organism; they found that the action of sulphadiazine is competitively inhibited by either *p*-aminobenzoic acid or *p*-aminobenzoylglutamic acid

but abolished noncompetitively by pteroic acid. They conclude that sulphadiazine must inhibit the synthesis of pteroic acid from *p*-aminobenzoic acid in this organism but were unable to obtain similar results in *E. coli*, *Staph. aureus* or *D. pneumoniae*. Gale (51) has shown that the condensation of glutamic acid into peptide and protein structures takes place only during growth in *Staph. aureus* and that this condensation is inhibited by sulphathiazole, although the effect may be an indirect one in this case. It is becoming evident that both *p*-aminobenzoic acid and glutamic acid play some key role in peptide formation in the growing cell.

The development of accurate micromethods of amino acid analysis has resulted in renewed interest in the amino acid composition of microorganisms (71, 72, 73). Stokes & Gunness (72) find that the amino acid composition of microorganisms is characteristic but may vary widely from organism to organism. If analyses are made in an attempt to study the protein of the organism, allowance must be made for the free amino acid concentrated within the internal environment of gram-positive bacteria and yeasts (73). Freeland & Gale (73) confirm the fact that the composition of an organism such as *E. coli* is independent of cultural conditions and find that, in general, gram-positive bacteria have a significantly higher arginine content than gram-negative species.

The toxins of *Cl. botulinum* and *Cl. tetani* have been purified and crystallized (74, 75, 76). Both have the properties of proteins, the former being a globulin with a molecular weight lying between 1×10^6 and 2×10^6. Synge and his colleagues have continued their studies of the structure of the polypeptides excreted by *B. brevis*. Gramicidin consists of minimum units containing 6 *d-leucine*, 6 *l*-tryptophan, 5 valine, 3 alanine, 2 glycine and 2 ethanolamine residues (77, 78). The valine is partially racemized and analysis of a partial hydrolysate of gramicidin has shown the presence of *l*-valyl-glycine in yields sufficient to account for more than half of the glycine present (79). Gramicidin S consists of a minimum unit containing one residue each of *l*-ornithine, *l*-proline, *l*-valine, *l*-leucine and *d*-phenylalanine (80). There are no free carboxyl groups and one free amino group which Sanger (81) has shown to be the δ-amino-group of ornithine. It follows that gramicidin S must consist of a cyclopeptide in which the repeating unit is (82)

α-*l*-valyl-*l*-ornithyl-*l*-leucyl-*d*-phenylalanine-*l*-prolyl-.

Christensen (83, 84) has carried out investigations into the struc-

ture of tyrocidine and concludes that, since the free groups consist of the δ-group of ornithine and the phenolic group of tyrosine with no evidence of free terminal amino or carboxyl groups, this substance is probably also a cyclopeptide.

Degradation of Protein

Proteolysis.—The literature on bacterial proteases up to 1943 has been reviewed by Maschmann (85). Chopra (86) has shown that the thermophilic bacteria, *B. thermophilus*, *B. aerothermophilus* and *B. thermoacidurans*, produce proteinases resembling tryptases in that they will hydrolyze gelatin, casein and denatured albumin but have little action on native albumin, and have pH optima ranging from 7.6 to 8.2. The enzymes are intracellular and pass into the medium only after autolysis of the organisms (87). Elliott (88) has shown that Group A streptococci produce a proteolytic enzyme under certain cultural conditions and that this enzyme, in addition to attacking casein, gelatin and benzoyl-*l*-arginineamide, attacks the type specific M antigen. Consequently organisms which are grown under conditions in which they produce the enzyme lack the type substance and cultures which produce the M substance are unable to form enzyme. The enzyme is activated by sulphydryl compounds and apparently resembles papain in its general properties. Roulet & Zeller (89) have shown that the tubercle bacillus produces peptidases which will hydrolyze glycyl-*l*-tyrosine, glycyl-*l*-leucine, *l*-leucylglycine and *l*-leucylglycylglycine. These enzymes are activated by manganese.

Degradation of amino acids.—Bacteria attack amino acids either by deamination or decarboxylation according to the pH of the medium during growth (90). Stumpf & Green (91) have shown that *Proteus vulgaris* possesses an *l*-aminoacid oxidase which oxidatively deaminates eleven of the naturally occurring amino-acids to the corresponding keto acids. The intact cells attack twenty-two amino acids so other enzymes must be present in the cell. A similar oxidative deaminase was found in *Aerobacter* and *Pseudomonas* species but not in *E. coli* or *Salmonella paratyphi*. Binkley (92) has obtained cell-free preparations of serine dehydrase and cysteine desulphurase from *E. coli*, and has shown that these enzymes can be inactivated by dialysis and then reactivated by the addition of certain metallic ions; serine dehydrase is reactivated by zinc, and cysteine desulphurase by zinc, magnesium, or manganese. The

product of the enzymatic breakdown in both cases is pyruvic acid and the results are in general agreement with the findings of Desnuelle *et al.* (93, 94, 95) who carried out a series of investigations of the specificity and properties of "cysteinase" and serine deaminase during the war years.

The breakdown of tryptophane has received considerable attention again during recent years. Krebs *et al.* (96) showed that *E. coli* can form indole from *o*-amino-phenyl-ethyl-alcohol but the kinetics of this reaction and the fact that it is inhibited by dimedon make it unlikely that it takes part in the production of indole from tryptophane (97). The work of Tatum & Bonner (30) on the synthesis of tryptophane from indole and serine by *Neurospora crassa* led to the suggestion that the reverse reaction is responsible for the production of indole from tryptophane by bacteria. In this case the other product of the breakdown would be *l*-serine though Dawes *et al.* (98) have produced evidence that *l*-alanine and not *l*-serine is produced. Either reaction would account for the finding of Woods (99) that the oxidation of tryptophane to indole is accompanied by an uptake of five atoms of oxygen per molecule of indole produced, if such oxidation is involved in the further degradation of either alanine or serine. It is not obvious in either case why the production of indole should be a strictly aerobic reaction. The breakdown of amino acids by decarboxylation and the properties of the enzymes involved have been reviewed in detail recently by the author (100).

A survey of the amino acid breakdown by *Aspergillus niger* has been published by Gorter (101). Mats of the organism deaminate glycine, alanine, *l*-glutamic acid, *dl*-leucine, *dl*-isoleucine and *dl*-aspartic acid. The deamination of aspartic acid occurs optimally at pH 2 to 4 and is accompanied by an uptake of oxygen. The enzyme concerned is not aspartase or a specific oxidase and is not specific for either isomer. Aspartic acid disappears from the medium some hours before ammonia formation occurs so that the amino acid is probably assimilated by the mould before being metabolized. It was not possible to demonstrate the presence of transaminase.

It is relevant to the present review to enquire what function, if any, these deamination and decarboxylase mechanisms have with regard to protein synthesis and growth. A number of facts can be taken into account in such a consideration: the deaminases

and decarboxylases are adaptive enzymes (100) and are formed to a minimal extent when an organism such as *E. coli* is growing in a synthetic medium containing no amino acids or protein products other than ammonia; the formation of deaminases is suppressed when rapid growth is taking place in the presence of fermentable carbohydrate (102); the formation of deaminases and decarboxylases takes place in the final stages of growth (102); the formation of deaminases takes place only in an alkaline growth medium while that of decarboxylases takes place in an acid medium and when this fact is taken into account together with the variation with pH of the activity of the enzyme units themselves, we find that there is a point approximately in the middle of the pH range where neither deaminase or decarboxylase systems are significantly active; in general those organisms which have wide synthetic activities also possess a variety of mechanisms for the degradation of amino acids while highly exacting organisms have few or no enzymes which are catabolic towards amino acids. It seems to the author that these facts are incompatible with the hypothesis that these mechanisms are primarily concerned with any growth process. The appearance of decarboxylases in response to an acid growth environment and towards the end of the growth-phase suggests that they act as neutralization mechanisms (102) and a similar function can be attributed to the deaminases. The gram-positive cocci are highly exacting towards amino acids and it is interesting that the only amino acid they normally attack is arginine (103) by the action of arginine dihydrolase. In this case the organisms attack an amino acid which is an essential nutrient; the enzyme concerned is formed optimally during the early phases of growth (104) and does not appear to function as a neutralization mechanism. It has been suggested (104) that the function of this breakdown process is to supply carbon dioxide to the organism since this is essential for growth and is not obtained by the normal homolactic fermentation of cocci. Further, in the presence of carbon dioxide, the organism is exacting towards ornithine and not arginine. This work suggests a further function of the deaminases and decarboxylases of gram-negative organisms: these organisms are nutritionally exacting towards both ammonia and carbon dioxide and it is possible that such enzymes are produced, in the presence of their substrates, as a means of supplying these simple nutrients. The formation of these enzymes is stimulated by envir-

onmental conditions which tend to reduce the natural occurrence or retention of such nutrients.

Many of these suggestions are frankly speculative and are put forward in the belief that the time has come for the microbiologist to concern himself seriously with the organization of biochemical events within the living cell, and to attempt an integration of facts learned from enzyme and metabolism experiments into an understanding of the cell as a biological unit rather than as an interesting safe-deposit for enzymes.

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INDUSTRIAL FERMENTATIONS

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It is impossible, in this review, to cover all phases of all industrial fermentations. Only fermentations of present actual or potential industrial significance will be mentioned. No attempt will be made to discuss nonmicrobiological aspects, such as recovery processes. Investigations dealing primarily with microbial metabolism will not be discussed. Only a few of the papers which have appeared during the war period can be mentioned; a complete bibliography would be inordinately long.

Ethyl Alcohol Fermentation

Ethyl alcohol production from grains.—During the war, the need for alcohol for butadiene production and other war purposes brought about an increase in industrial alcohol production in the United States from 299 million proof gallons in 1941 to 988 million in 1944 (1). A large part of the wartime alcohol was produced from grains, especially wheat. Stark, Kolachov, & Willkie (2) report yields of from 4.94 to 5.8 proof gallons per 56 lb. bushel of dry grain from various wheats, as compared with 5.9 to 6.1 for corn. Yields of the same magnitude are reported by Thorne *et al.* (3). Much use was made of crude granular wheat flour for alcohol production. This material, containing all of the wheat carbohydrate, was advantageous for use in plants designed for molasses operation, since a minimum of new equipment was necessary for its use. Erb & Hildebrandt (4) have reported plant yields, in plant facilities designed for molasses operation, of eleven proof gallons per one hundred pounds dry grain. When mold (see below) was substituted for malt, higher yields were obtained.

A recent logical development in grain-alcohol production is the use of continuous conversion. In classical practice, the mixture of cooked grain and malt were held at 60°–65°C. for from thirty to sixty minutes for conversion of starch to dextrins and maltose. Inasmuch as considerable amylase destruction takes place during the conversion period, and since a large part of the conversion actually takes place in the fermenter during the forty-eight to seventy-two hour fermentation period, a procedure involving a much shorter conversion period would appear advantageous. The advantages have been pointed out by the Seagram group (5, 6).

They report a 2 per cent increase in alcohol yield when a one-minute conversion time is used. The economic advantages of continuous conversion, when combined with modern continuous cooking procedures, are obvious.

The wartime malt shortage stimulated interest in amylase sources other than malt. Much work has been done on mold bran as an amylase source. Mold bran is prepared by growth of a mold on moist acidified wheat bran. Hao, Fulmer, & Underkofler (7) investigated twenty-seven mold strains, and found two strains of *Aspergillus oryzae*, one of *Rhizopus delemar*, and one of *Rhizopus oryzae* which yielded mold bran giving maximal alcohol yields when used at a level of 4 per cent of the grain. Underkofler, Severson, & Goering (8) summarize previous work, and report plant-scale tests of mold bran, alone and together with malt, as a saccharifying agent in grain fermentations. The data show mold bran (at a 4 per cent level) to give yields equal to or slightly better than the yields obtained with malt. Since, according to the authors, mold bran can be produced much more cheaply than its malt equivalent, the use of mold bran results in reduced costs. These authors also found that with the yeasting procedure used, the use of mold bran in the yeast tubs was very advantageous. In older work (9, 10), in which the use of mold bran increased alcohol yields as much as 12 per cent, the malt control fermentations gave lower yields than are generally obtained with malt. When the malt comparison fermentations are conducted under optimal conditions, as in more recent work, the yield increase due to the use of mold bran is much smaller.

Hao & Jump (11) have used a wide variety of microbial amylase preparations as converting agents, and find that they are in general more active than malt, giving maximal alcohol yields at lower levels. Erb & Hildebrandt (4) have used mold amylase prepared by submerged growth for conversion of granular wheat flour mashes on a plant scale. The molds were of the type used in the amylo process, *Rhizopus boulard* and *Rhizopus delemar*. The mold was grown in tanks with aeration on a medium containing stillage, granular flour, malt, and salts. The use of mold together with 2 per cent malt gave average alcohol yields of 12.1 proof gallons per one hundred pounds dry grain, compared with 11.0 proof gallons with malt. The yield with malt represents only 84 per cent fermentation efficiency (the plant was apparently not designed for grain operation). Hence, as in the earlier work on mold bran, fungal converting agents appear to be less affected by un-

favorable conditions than is malt. It has been pointed out by Stark (12) that current work in a number of laboratories on production of microbial amylases by submerged fermentation is of great interest for another reason. The producer of alcohol from grain is, at present, forced to add to a sterile cooked grain mash a heavy load of contaminating bacteria by the addition of malt or mold bran. An amylase produced in the distillery by submerged fermentation under aseptic conditions could be added to the mash without introducing contamination, and would make possible aseptic distillery operation.

Production of ethyl alcohol from wood products.—Before the war, cane blackstrap molasses, at a cost of approximately one cent per pound of fermentable sugar, was the carbohydrate source of choice for industrial alcohol production. During the war, a wide variety of materials, mostly grains, were used, since availability rather than cost was the prime consideration. At this writing, the cost of fermentable sugar in the form of blackstrap molasses is about three cents per pound.

The costs of producing alcohol from various raw materials have been estimated by Jacobs (1) and by Tousley (13). The cost of alcohol production from wood sugar is discussed by Harris & Beglinger (14). A significant feature of recent comparative cost estimates is the emergence of such carbohydrate sources as sulfite waste liquor and wood sugar in an extremely favorable position. Thus, it is estimated (14) that alcohol can be produced from waste wood at a cost of 28.4 cents per gallon, and from sulfite waste liquor (13) at a cost of less than twenty cents. Such figures, being a fraction of the present cost of production from molasses, make it apparent that present interest in fermentation processes involving these materials is justified.

Harris and co-workers (14, 15, 16) have developed modifications of the Scholler process of wood hydrolysis which yield from fifty to sixty gallons of alcohol per ton of wood, at an estimated cost (14) of 22.3 to 28.4 cents per gallon when Douglas fir is used. A plant using the process (17) is at present in operation at Springfield, Oregon. It has a designed capacity of 10,700 gallons of 190-proof alcohol per day. Recent work has shown that operation of only four of the ten 70,000 gallon fermenters will give the designed performance. This plant thus follows the general rule that plants for new fermentation processes invariably are found to be overdesigned in fermentation capacity.

Problems involved in the fermentation of wood hydrolyzates have been studied by Harris *et al.* (16) and by Leonard & Hajny (18). In general, the methods used involve adding large yeast populations (one or more volumes per cent of yeast cells) to lime-neutralized hydrolyzate containing nutrient salts. The yeast is re-used. Cane molasses or malt sprouts are used as nutrients in growing the inoculum. The time necessary to ferment a five per cent wood sugar solution varies with the yeast population and the type of hydrolyzate used. One per cent yeast in Douglas fir hydrolyzate gives a ten to fourteen hour fermentation. Shorter fermentation times were obtained in German wood sugar alcohol plants during the war (19, 20). By a continuous fermentation in several tanks arranged in series, with the yeast cells centrifuged from the effluent being continuously added with the feed to the first tank, extremely high yeast populations and fermentation times of four to six hours were achieved. In one plant, the yeast was held at pH 2 to 2.5 for two hours once each month to kill contaminating bacteria. Alcohol yields were apparently a little under fifty gallons per ton of wood.

The production of alcohol from sulfite waste liquor, which has long been commercial practice in Europe, has not been economically feasible in the United States until recently. The present high price of fermentable carbohydrates, together with the increasing stream pollution problem of the paper industry, has stimulated interest in this fermentation. One full-scale plant is in operation at present (21). The liquor is prepared for fermentation by removal of sulfur dioxide by steam stripping and addition of urea. The fermentation is continuous, taking place in eight 80,000 gallon fermenters arranged in series. The yeast, separated from the effluent beer in yeast centrifuges, is returned to the first fermenter. A yeast content of 0.5 to 1 volume per cent is maintained, and the fermentation time is twelve to twenty hours. The daily capacity is 6,000 gallons of alcohol.

There has apparently been no recent interest in glycerol production by yeast fermentation, although a number of patents [e.g., (22, 23)] have recently appeared.

Production of Yeast

Bakers' yeast.—The most interesting recent development in the field of bakers' yeast production has been the large-scale production of active dried yeast. During the war, enormous amounts of

this material were required by the armed forces for overseas use. The active dried yeast now on the market has roughly 80 to 85 per cent of the activity, on the dry basis, of a good fresh bakers' yeast, and keeps for months without refrigeration. According to Merritt & Stamberg (24) active dried yeast is more economical than fresh yeast.

De Becze & Liebmann (25) have reviewed methods of aeration used and proposed for bakers' yeast production. They conclude that the problem is far from solved, but that the use of porous substances and mechanical devices for air dispersion are the most promising aeration techniques.

Food yeast.—Thaysen *et al.* (26, 27, 28) have developed a process for continuous production of food yeast from molasses. The process was designed for use in Jamaica, the yeast to be added to the flour consumed on the island as a vitamin and protein dietary supplement. Two new strains of *Torulopsis utilis* were developed. One (*var. major*) has a large cell size, which facilitates filtration or centrifugation operations. The other, intended for tropical use, grows well at 39°C., thus simplifying the cooling problems that arise in locations where low-temperature cooling water is not available. In pilot plant work, it was found possible, on a molasses-phosphate-ammonia medium containing 4.5 per cent sugar, to carry out a single-stage continuous fermentation without the loss of appreciable carbohydrate in the effluent. In a holding time of four hours, a yield of dry yeast equal to 60 per cent of the added sugar was obtained.

In conjunction with the development of wood sugar alcohol processes, there has been considerable work on the production of food yeast from wood hydrolyzates. Peterson, Snell & Frazier (29) found that the yields of *Torula utilis* or *Candida tropicalis* on wood sugar from the hydrolysis of a large number of wood species varied from 30 to 42 per cent of the reducing sugar present in the hydrolyzate. On Douglas fir, spruce, and southern yellow pine hydrolyzates, yields were between 37 and 40 per cent. For fermentation, the hydrolyzates were neutralized to pH 5 with calcium carbonate. Urea and phosphate were added. Sixteen hours were required to ferment 1 per cent sugar solutions when an inoculum of 1 gm. of yeast per liter was used. With larger inocula, fermentation was more rapid. Kurth *et al.* (30, 31) investigated the production of *Torula* cells from wood sugar ethyl alcohol stillage. The stillage contains the pentoses of the wood sugar, some acetic acid, and

small amounts of other compounds. The reducing sugar content varied from 0.6 to 1.5 per cent. Yields of 50 to 60 per cent of the reducing sugar were obtained, substances other than sugar being also fermented. The fermentation required 18 to 24 hours. Considerable reduction in biological oxygen demand was observed. It should be emphasized that the slow fermentations obtained in many laboratory studies can be accelerated when equipment is used which is capable of providing an adequate air supply for large yeast populations.

Because of the increasing acuteness of the sulfite waste liquor disposal problem, there is much interest in production of yeast from sulfite waste liquor. Since *Torula* will use pentoses and organic acids as well as hexoses, a large part of the biological oxygen demand of the liquor can be removed by the fermentation. If the yeast can be sold on the feed market at a price that covers the cost of production, the process would be an excellent method of waste disposal. If efficient means of utilizing the lignin residue are also found, additional advantages result. A $400,000 plant is to be built (32) at Rhinelander, Wisconsin, as a full-scale test of the *Torula* process. One unknown factor is the extent to which the market price for yeast would decrease under the impact of large-scale food yeast production from sulfite waste liquor.

Yeast for human food was produced extensively during the war in Germany from sulfite waste liquor and wood sugar (19, 20), approximately 16,000 tons being produced in 1944. In general, high populations of *Torula* were carried in continuous or batch fermenters. Yeast production rates were as high as 2.2 gm. of yeast per hour per liter of medium. This compares with 6.75 gm. reported by Thaysen (27) from molasses, a much better growth medium, and with 14 gm., obtained by Feustel & Humfeld (33) in a small laboratory fermenter. The latter workers estimated their yields from cell volume measurements.

An interesting German development is the Waldhof fermenter (19, 20). The cylindrical fermenter, 7.5 m. in diameter and 4.5 m. high, contains a central draft tube 1.25 m. in diameter and 3 m. high, raised one meter from the bottom of the fermenter. Below the draft tube air enters the medium through a revolving aerator, and rises through the medium outside the draft tube. The resulting foam falls through the draft tube and is recirculated by the centrifugal action of the aerator. Because of recirculation of the emulsion, air requirement is low and antifoam agents are not necessary. The power requirement is, however, fairly high.

Antibiotics

Only two antibiotics, penicillin and streptomycin, are at present in industrial production. Only fermentation problems involved in their production will be discussed here. A general discussion of penicillin and streptomycin is found in the review on antibiotics.

Penicillin.—The production in 1946 of 25.8 trillion units of penicillin (more than 1,000 times the 1943 production) gives the penicillin fermentation a position, in value of product, second only to the ethyl alcohol fermentation. Industrial production of penicillin is carried out in aerated and agitated media, fermenter capacities ranging from 2,500 gallons to 10,000 gallons. Rigid asepsis is essential, since contaminating organisms usually produce penicillinase. The *Penicillium* strains used are mentioned in the review on antibiotics. Unfortunately, no detailed descriptions of commercial penicillin fermentation installations have been published. Stefaniak *et al.* (34, 35) have described the construction, operation, and performance of pilot plant penicillin fermentation equipment. Various modifications of the steep liquor-lactose medium of Moyer & Coghill (36) were used in 100 gallon tanks, seeded with ten per cent by volume of a mycelial suspension from a seed tank. Maximum penicillin yields were reached after sixty to seventy-five hours at temperatures between 23° and 29°C. Yields varied greatly with the culture, medium, and aeration rate used. Highest yields were obtained [Gailey *et al.* (37)] with strain Q176, which averaged 761 units per ml. This yield could be increased to 1,000 units (38) in the presence of phenylacetic acid. Air requirements for the fermentation vary with the size of the fermenter and the design of the air dispersion equipment. With culture X1612, carbon dioxide production rates as high as one volume of carbon dioxide per minute per one hundred volumes of culture are observed (34, 39). The theoretical air requirement for this metabolism rate would be in the neighborhood of five volumes of air per one hundred volumes of culture per minute, but an aeration rate of twenty times this magnitude was required to maintain high metabolism rates. It was concluded that adequate air dispersion was not being obtained. Yields obtained in commercial production of penicillin have not been published. Methods of purification of penicillin from fermentation beer have been described by Whitmore *et al.* (40).

Streptomycin.—A number of companies are producing streptomycin on a commercial scale. The general procedures used in the

$3,500,000 plant of Merck and Company are described by Porter (41) and by Silcox (42). The medium used is not described, although obvious modifications of the shake flask medium of Waksman *et al.* (43) are discussed. From the number of seed stages used, it is apparent that a heavy vegetative inoculum is used in the final 15,000 gallon fermenters. The aeration level used is of the order of twenty-five volumes per one hundred volumes of medium per minute. Agitators are used to aid in air distribution. The fermentation temperature is between 25° and 30°C. The general outline of the recovery procedure used is given. The capacity of the plant is 100 kg. of streptomycin (calculated as pure free base) per month.

Bacterial Fermentations

Production of 2,3-butylene glycol.—There is at present no industrial production of 2,3-butylene glycol or acetoin. However, the possibility of the use of the diol for conversion to butadiene stimulated considerable laboratory and pilot plant research on its fermentative production. A number of fermentation and recovery processes have been developed, and if a market for either the glycol or acetoin develops, these compounds can be produced at low prices. The diol is produced from hexoses and pentoses by members of the genus *Aerobacter*. Under anaerobic conditions, considerable amounts of ethyl alcohol are produced, but under moderate aeration, alcohol production is suppressed, and yields as high as 40 gm. glycol per 100 gm. sugar can be obtained. With high rates of aeration yields are lower and acetoin production is higher. Acid-hydrolyzed grains (44), acid-hydrolyzed starch (45), wood sugar (46), and other carbohydrates may be used.

Acetoin is readily obtained from 2,3-butylene glycol by oxidation with *Acetobacter suboxydans* [Fulmer *et al.* (47)]. Only the levo and meso forms are attacked [Underkofler *et al.* (48)]. Since *Aerobacter* produces predominantly the meso form, it is possible to convert the glycol in fermented *Aerobacter* beers to acetoin by inoculation with *Acetobacter* and incubation with aeration. Sjolander & Eisenman (49) obtain 80 per cent conversion in beers containing more than 6 per cent glycol. This process appears to afford a feasible method of large-scale acetoin production.

Aerobacillus polymyxa will ferment starches with the production of levo 2,3-butylene glycol and ethyl alcohol. Production of 2,3-butylene glycol from grain mashes by this organism has been extensively investigated. Among the more recent papers are those

of Adams *et al.* (50 to 53). Approximately ten pounds of glycol and six pounds of alcohol are obtained per bushel of wheat in pilot plant operation. Fermentation of 15 per cent wheat mashes requires ninety-six hours, but the time can be shortened by aeration with an inert gas or operation under reduced pressure.

A number of methods (54 to 58) have been proposed for recovery of butylene glycol from fermentation beers. They will not be discussed here.

Lactic acid fermentation.—Peckham (59) has described the usual commercial processes of lactic acid production. The organisms used (60, 61) may be either *Lactobacillus* species or certain members of the genus *Bacillus*. The latter organisms produce the *l*(+) acid, while the lactobacilli may produce either enantiomorph. The use of high temperature organisms is advantageous in overcoming contamination. Normal commercial practice does not involve strict asepsis. Sugar sources may be crystalline glucose, whey, or molasses, depending on the recovery process used and the purity desired in the product. Calcium carbonate is present in excess during the fermentation and continuous neutralization is effected by gentle agitation. Recovery processes are outlined by Peckham (59). Filachione & Fisher (62) have developed an interesting recovery process based on methylation of crude aqueous lactic acid and continuous distillation of the resulting ester in methyl alcohol vapor.

Leonard & Peterson (63) have described a process for the production of lactic acid from sulfite waste liquor, based on a *Lactobacillus* fermentation followed by solvent extraction of the acid. The process, if it proves practical on a large scale, is of interest because of the relatively enormous quantities of lactic acid potentially available; between fifty and one hundred million pounds per year if only ten per cent of the present sulfite waste liquor production is used.

Clostridium fermentations.—During the war period, American butyl alcohol producers were forced to return to grain as a carbohydrate source. Fortunately (since grain had been used exclusively before the discovery of efficient molasses-fermenting organisms), suitable cultures and ample "know-how" were available.

A number of patents [e.g., (64, 65)] have been granted on the use of new organisms in butyl fermentation, but little has been published recently on the technology of the fermentation. Pan (66) finds that the addition of unheated mold bran to sugar mashes accelerates the butyl fermentation. Unless the mold bran used were

free from contamination, however, its use would appear dangerous. There has been considerable interest in France [Depasse (67, 68)] in a process involving fermentation of molasses or other crude carbohydrate material with a mixed anaerobic acid-producing flora, followed by conversion of the resulting calcium salts of aliphatic acids to a mixture of ketones. The ketone mixture is said to be a valuable antiknock adjunct to motor fuel.

Other Fermentations

Citric acid.—The most interest in the field of citric acid production is in recent work on submerged citric acid production. As industrial use of aerobic organisms in submerged culture processes increases, it appears very possible that cultures, media, and techniques will be developed which will make commercial production of citric acid by submerged fermentation economically feasible. The submerged growth procedures described by Szucs (69) and by Waksman & Karow (70, 71) involve growth of the mold mycelium on one medium, followed by citric acid production on another. Such procedures might occasion difficulty when attempted aseptically on a commercial scale. The long time cycles required in known citric acid processes would result in an inordinate requirement for fermenter capacity and aeration facilities. A heavy mycelial growth, desirable from the standpoint of fermentation rate, is undesirable in that too large a proportion of the sugar is required for mycelium production. Another unknown factor is the ultimate postwar price of the various carbohydrate sources that might be considered for citric acid production.

There have been a number of recent papers on factors involved in the production of citric acid by the surface growth method. Perlman, Dorrell, & Johnson (72) studied the effect of various metallic ions on citric acid formation from sucrose. Optimal yields (60 to 70 gm. citric acid monohydrate per 100 gm. added sucrose) were obtained when 0.1 mg. iron or 0.1 mg. manganese per liter was added. The effect of metals varied somewhat with the strain of *Aspergillus niger* used. They find that the materials in commercial sucrose responsible for low citric acid yields are cations, and can be removed by cationic exchange treatment. Gerhardt, Dorrell, & Baldwin (73) report yields of 50 gm. monohydrate from 100 gm. of sugar in ferro- or ferricyanide treated beet molasses media. Perlman, Kita, & Peterson (74) find that a large part of the material in cane molasses responsible for low citric acid yields is

present in the ash. Eisenman & Blumenfeld (75) find the N to K ratio to be important in molasses citric acid media; they specify a ratio N:K of 1:1.35 to 1.5. Von Loesecke (76) has reviewed mycological citric acid production. The operation of a German citric acid plant, employing surface growth on beet molasses media, has been described by the British Intelligence Objectives Subcommittee (77, 78). The diluted molasses (30 per cent sugar) is brought to pH 7.5 with sulfuric acid, heated to 80°C., treated with 10 per cent excess potassium ferrocyanide to remove iron, treated with one gram of phosphoric acid per kilogram of molasses, boiled for ninety minutes, and diluted to 15 per cent sugar. The fermentation is carried out in aluminum pans containing an 8 cm. depth of medium. Spores for inoculation are blown into the fermenter room with the air supply. After ten days incubation at 30°C., a citric acid yield of 70 per cent of the original sugar is obtained. The strain of *Aspergillus niger* used produces no oxalic acid unless the temperature exceeds 32°C.

Other acids.—Itaconic acid, which is produced by *Aspergillus terreus* in surface culture on sugar media, is of interest as a raw material for plastics. Lockwood & Ward (79) have reported experiments with a superior strain of this organism. The yield of itaconic acid is 30 per cent of the added sugar in 15 per cent glucose solutions. The raw material cost (based on glucose at 5 cents per pound) is 22 cents per pound of itaconic acid. Kane, Finlay, & Amann (80) report yields of 25 to 28 per cent in 10 per cent sugar solutions.

Williams (81) has described the operation of a commercial gluconic acid plant.

Riboflavin.—The first organisms to be used for commercial riboflavin production were butanol-producing members of the genus *Clostridium*. Recently, however, other organisms have received attention. Burkholder (82) found that *Candida guilliermondia* is a high flavin producer. Tanner, Vojnovich, & Van Lanen (83), studying the same yeast, found that trace metals had a large effect. They obtained yields of 120 μg. of flavin per ml. in the presence of 0.5 to 1 μg. of iron per 100 ml. of medium. More than 200 μg. of flavin per ml. of medium were yielded by *Candida flareri*.

It was observed in 1935 by Guilliermond *et al.* (84) that *Eremothecium ashbyii* produces a pigment related to flavin. This was isolated in 1938 as riboflavin by Mirimanoff & Raffy (85, 86).

Schopfer (87) showed that the organism required for growth biotin, thiamin, and inositol, in addition to uncharacterized constituents of peptone. He found the addition of peptone stimulates flavin production by the fungus.

Probably most of the microbiological riboflavin being produced at present is synthesized by *E. ashbyii*, grown in submerged, aerated culture. None of the producers have published data on yields, but they are believed to be very high, although Rudert (88) reports only 6,000 μg. per gm. of dried fermentation beer. Hines (89, 90) describes the use of chemicals or microörganisms to reduce the flavin in the fermented culture, the reduced flavin being more insoluble. The liquor remaining after filtering off the reduced flavin contains only 28 μg. flavin per ml.

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QUATERNARY AMMONIUM COMPOUNDS

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The quaternary ammonium compounds (called quats in this discussion for the sake of brevity) are characterized by their property of greatly depressing the surface tension of water. This property puts them in the group of chemicals variously described as wetting agents, detergents, emulsifiers, or dispersing agents. In that group of surface-active agents, we must distinguish between anionic detergents of acid nature, and cationic detergents, or substituted ammonia compounds. In addition, there are a few neutral surface-active agents. Only the cationic detergents or quats are discussed in this review.

Fundamental Studies

The first observations of germicidal properties in this group were made in 1916 by Jacobs and associates (1, 2, 3) who studied the relation between structure and bactericidal effects in the hexamethylene tetramine group. This was followed in 1928 by Hartmann & Kägi's work (4) with other quaternaries. However, the great possibilities of this class of compounds were not realized until Domagk (5) in 1935 reported on the long-chain quaternary ammonium salts. He found that germicidal properties were possessed by all quaternary ammonium salts in which at least one radical was a long-chain aliphatic group (C_8H_{17} to $C_{18}H_{37}$), whether plain or substituted. Two or even three N-valencies may serve as part of a cyclic compound as in hexamethylene-tetramine, pyridine, isoquinoline, etc., and the anion may be halide, sulfate, acetate, or other similar group. Domagk's report opened up a wide field of exploration and has resulted in a rapidly growing literature, both in this country and abroad.

Constitution and germicidal action.—Domagk (5) found notable germicidal action only when at least one of the four radicals had a long carbon chain. He worked mostly with Zephirol which is alkyl dimethyl benzyl ammonium chloride. The alkyl group could be a chain containing from eight to eighteen carbon atoms. The same compound is sold in the United States as Zephiran, Roccal, or

BTC. Detailed data on many similar quaternaries are given by Valko & DuBois (6).

Compounds of the Hyamine group contain two radicals with benzene rings (7). When cyclic nitrogenous compounds are used, only one radical remains to be substituted. Shelton (17, 18, 19) found that with pyridine, the cetyl radical conferred strongly germicidal properties. This compound (cetyl pyridinium chloride) has the trade name Ceepryn. Some of the Emulsept products are also substituted pyridines. Shelton also studied the germicidal efficiency of other substituted cyclic amines, such as picolin, lutidine, quinoline, piperidine, pipecoline and morpholine.

Limitation of space prohibits any discussion of the relationship between chemical structure and germicidal efficiency. This relationship in homologous series has been recently correlated with thermodynamic activity (20). This theory has not been applied as yet to quats. [For details on homologous series, see (6 to 16, 41).]

In the following discussion, trade names will be used for the sake of simplicity and shortness. The most commonly used quats mentioned below are:

CTAB (Cetavlon) = cetyl trimethyl ammonium bromide

Zephiran = Roccal = BTC. = alkyl dimethyl benzyl ammonium chloride

Phemerol = Hyamine 1622 = Polymine D = p-tert. octylphenoxy ethoxy ethyl dimethyl benzyl ammonium chloride

Ceepryn = cetyl pyridinium chloride.

Toxicity.—Many experiments with a great diversity of quats have led to the unanimous conclusion that they are not toxic to animals in the concentrations in which they are commonly applied. A summary of these investigations was recently given by Valko (36).

Bacteriostasis.—When Dubos wrote [(21), p. 290]: "the difference between bacteriostatic and bactericidal effect is often of a quantitative rather than of a qualitative nature," he expressed a common conception [see also (22, 23) and others]. However, bacteriostasis is treated here separately from bactericidy because (a) with gentian violet bacteriostasis and bactericidy are quite different reactions (24), (b) Roberts & Rahn (25) observed complete enzyme inactivation at bactericidal concentrations but not at bacteriostasic doses, and (c) many very efficient bacteriostats do not kill bacteria even at very high concentrations.

Bacteriostasis may be due either to reversible inactivation of enzymes, or to reversible disruption of semipermeable membranes, or to reversible interference with the mechanisms of synthesis or cell division.

The relation between enzyme inhibition and bacteriostasis has rarely been studied. Sevag (26) found that 18 p.p.m. Zephiran retarded growth of yeast cells 65 to 83 per cent whereas the oxygen uptake was either not affected at all or even increased as much as 50 per cent. With 5 p.p.m., a 34 per cent decrease of growth was accompanied by a 60 per cent increase of oxygen uptake. This agrees with data by Roberts & Rahn (25) who tested *Escherichia coli* under conditions where energy could be obtained only through oxidase or dehydrogenase systems. These enzymes were not affected by concentrations of Zephiran which inhibited growth completely. Bacteriostasis must be caused by some other reaction. Semipermeability of the membranes cannot very well be reversible, because during the period when the membrane is completely permeable, the cell contents would diffuse out and cause the death of the cell. That leaves as the most probable explanation of bacteriostasis the interference with synthesis, i.e., interference with the doubling of essential molecules such as enzymes or genes or other proteins, or with cell wall construction, or interference with the more mechanical aspects of cell division. The microscope does not reveal abnormal cell morphology.

Inhibition studies with gram-negative bacteria by Randles & Birkeland (27) suggest that inhibition is due to preferential adsorption of detergent cations at that site of the cell which normally adsorbs the necessary nitrogen compounds. No data are given to indicate whether this is a mechanism similar to that functioning in inhibitions elicited by sulfa compounds.

Recovery from injury or inhibition by quats is slower than with most disinfectants because the adsorption layer of quats on the cell surface is not readily removed, and therefore injury to the cell may continue for some time after the disinfectant has been removed from the medium by dilution, adsorption or even by antidotes. This interferes with the appraisal of germicidal efficiency. Transfer of the bacteria to new broth frequently does not permit reproduction, while transfer combined with actual removal of the adsorbed quat shows that the bacteria were not really dead (28, 29, 30). Chemical removal, by an anionic detergent, results in

more complete recovery than physical removal by addition of charcoal (31). The use of media containing soap (5), anionics (29, 32), or lecithin or other phospholipids (30, 32, 33) to remove adsorbed quats has been widely recommended [cf. p. 181].

The lethal reaction.—Various explanations have been advanced for the death of bacteria resulting from the action of quats, namely, disruption of the cell membrane, inactivation of enzymes, or inactivation of other types of indispensable proteins.

Enzyme inactivation which had been accepted for many years as the general explanation for all kinds of sterilization, has been assumed by several authors to be the cause of death by quats. Baker *et al.* (34, 35) found that, as a rule, complete sterilization is accomplished by concentrations which cause a great decrease of the oxygen uptake [see also (26, 43)]. However, in a few cases, loss of oxygen consumption is not accompanied by death, and any such exception makes the explanation rather doubtful. Roberts & Rahn (25) observed practically complete and irreversible inhibition of certain oxidase and dehydrogenase systems by lethal concentrations, but were in doubt whether this was the cause or the result of death.

The denaturation of enzymes within the cell requires the penetration of the cell membrane by quats. Valko [(36), p. 475] believed that "there is no reason to assume that the surface-active ions will not penetrate into the bacteria. Indeed, Kuhn & Jerchel (37) proved that surface-active ions penetrate into the living cell." Dubos (21) is of the same general opinion whereas Hotchkiss [(23), p. 490] wrote: "In the case of the usual surface-active agents, the experience of cell physiology would tend to predict that they could not penetrate the cell as long as it is alive." However, Dyar (49) observed that the cytoplasm of vegetative cells suspended in Ceepryn stained black with Sudan Black B, indicating that the quat had penetrated into the cell and that its hydrocarbon portion was stained by the fat dye.

Hotchkiss assumes that the quats first disrupt the cell membrane, and make it completely permeable, thereby markedly reducing enzyme activity and completely preventing multiplication, although morphologically, the cell appears unchanged. Hotchkiss supported his explanation with determination of the amounts of nitrogen and phosphorus compounds released from the treated

cells. Unfortunately, the experimental error of these measurements is very great.

Valko [(36), p. 478] believed in general protein denaturation as the lethal cause.

"Surface-active ions possess extraordinarily strong intrinsic affinity for proteins and combine readily with them. This combination causes disturbance of the intramolecular structure of proteins by upsetting the balance of the electrostatic forces as well as that of the non-Coulombic cohesion in the molecule. At the same time, the interaction of the proteins with the solvent molecules may undergo profound changes and the bonds between the components of the conjugated proteins may be disrupted."

The experiments on reactions of proteins with quats summarized by Valko [(36), p. 470] show, as a rule, that approximately equal weights must react to produce denaturation or precipitation of the proteins. In standard bacteriological technique, the culture, diluted 1:10, contains about 20 p.p.m. bacterial solids, and with the more efficient quats, about 20 to 100 p.p.m. are sufficient to kill in five to ten minutes. However, only a small fraction of the disinfectant in solution actually combines with the bacteria, so that death is probably not due to a general denaturation of all cell proteins. The necessity of general denaturation of all cell proteins appears improbable because of the logarithmic order of death (31) observed for 99 to 99.9 per cent of the cells. If death were due to a destruction of the surface, the order should be similar to that of multicellular organisms, as is the case with death by chlorine [(38), p. 93]. The survivor curves for quats and for chlorine bend in opposite directions. The initial logarithmic order with quats pertains to the single cells. The much slower death rate of the few remaining bacteria (40) is explainable by the clumping of the cells under the influence of quats (39).

In the opinion of Valko & Dubois (29), "the antibacterial behavior of surface-active cations is in agreement with that of toxic metallic ions and dye cations. They can be considered as a phenomenon of ionic exchange." The above refers largely to the explanation advanced by McCalla (45). The present authors hesitate to accept this analogy, because it has never been proved that quats act by their cations. Since most quats lose their efficiency almost completely in acid environment when they should be most completely dissociated, it may well be that their activity depends

primarily upon the undissociated molecules as is the case with many organic compounds (46, 47).

An interesting observation was made by Dyar & Ordal (44) when they studied the effect of surface-active compounds upon the electrokinetic mobility of different species of bacteria. As the Ceepryn concentration was increased, the negative electric charge of the bacterial cells remained constant at very low concentrations, but at about 10^{-5} molar, the charge decreased to zero, then became positive and finally became stable at concentrations between 10^{-4} and 10^{-3} molar. While these experiments were made only to study the nature of the cell surface, it is interesting to note that with *Staphylococcus aureus* and *E. coli*, the bacteria died rapidly when the Ceepryn concentration was just strong enough to produce a noticeable decrease in mobility. This suggests a relation between surface charge and disinfecting power which has also been claimed by Bradbury & Jordan (48).

The "reversibility" of the "killing "action of these agents by anionic detergents or other means (29) is probably not due to the reversion of the lethal reaction by removal of the quat, but more likely to a last-minute prevention of the lethal reaction which would have taken place in routine transfer, but was intercepted by removal of the quat. Physical removal, by adsorption, is slower in saving bacteria from death than chemical inactivation of the quat by anionics (31). All experiments, by all authors, have shown "recovery" of bacteria to be limited to a short time, about thirty minutes, after exposure; beyond that, cells are irreversibly killed.

Factors Affecting Germicidal Efficiency

As suggested above, surface-active agents are "temperamental." Their efficiency may be greatly decreased by conditions which hardly affect the so-called plasma-poison group of disinfectants. The quats are completely neutralized by anionic compounds, including the soaps, and by phospholipids, with which they form harmless compounds (5, 29, 32, 33, 57, 68, 73).

Adsorption.—Surface-active compounds are readily adsorbed and filter paper, charcoal, bentonite (50) and agar (51, 83) remove part or all of the dissolved quats from solution. So do bacterial surfaces (50); those of dead cells probably being as efficient as those of living ones. Precipitated proteins also adsorb quats. Ad-

sorption has its good and bad features in the practical use of quats.

Fat.—Fat affects quats physically. On fatty surfaces, they are adsorbed in oriented layers, the hydrophobic, organic end of the molecule is directed towards the fat surface, while the ⋮N—OH group faces the aqueous phase. As the great reactivity of quats depends upon the ⋮N—OH group, such oriented adsorption films have two different sides of which the one, directed towards the fat, is non-toxic whereas the other, directed towards the aqueous phase, is strongly germicidal (50). This explains the peculiar effect observed in the disinfection of hands with quats (52) [cf. p. 185].

Protein.—Proteins combine with quats chemically, frequently producing a precipitate (36, 67). The reaction requires approximately equal weights of proteins and quats, so that a quat solution of 1:1,000 may be made completely harmless by a 1 per cent protein solution. Quats are inefficient in milk (53, 54). In presence of 10 per cent serum, the killing concentration of Ceepryn had to be increased four to eight times with streptococci or staphylococci, and twelve to twenty-five times with enterobacteria (55). Hence, the ease with which they react with proteins influences greatly the efficiency *in vitro* and especially *in vivo* [cf. p. 180]. Gelatin has been found to be an exception (70).

pH.—Acidity decreases the efficiency of many quats so decidedly that they are completely inefiicient at pH 3, and only weakly bactericidal at pH 4 (42, 55, 56, 57). The only exception known so far is Ceepryn which is as efficient at pH 3 as at pH 8 (53, 55).

Temperature.—Temperature affects the action of this group as it affects all other disinfectants. An increase in temperature increases the efficiency, the temperature coefficient between 20° and 37°C. being less than 2 (55). Contradictory or variable results (62, 63) may be obtained at higher temperatures because it is sometimes overlooked that at the optimal temperature, bacteria are multiplying at the fastest possible rate which is a dangerous rate, and that a relatively slight disturbance may cause death. That would result in a seemingly abnormal temperature coefficient.

Species.—Generally speaking ,the quats are not very selective in their efficiency (35, 58, 59, 60, 62, 63, 64). Commonly, somewhat larger doses are required to kill the gram-negative enterobacteria

than streptococci or staphylococci, but rarely more than double the amount (55). A striking exception is *Pseudomonas aeruginosa* for which ten to twenty times as much is needed as for staphylococci (55). *Mycobacterium tuberculosis* (65) and *M. phlei* (55) are even more resistant. Pathogenic fungi are somewhat more resistant than the gram-negative bacteria.

Bacterial spores are not easily killed. General statements which give no description of technique and no quantitative data (63, 66) are not convincing. The high concentrations required may produce bacteriostasis in the transfer tubes, unless special precautions are taken, and may simulate sterilization. According to DuBois & Diblee (40), a 1:10 dilution of Tetrosan is needed to kill all spores in a culture of *B. metiens* although a 1:5000 dilution kills 60 to 75 per cent almost immediately [see also (94, 95)].

Potentiation.—When two different disinfectants are applied simultaneously, it must be expected that their combined effect is greater than that of each disinfectant alone. The term potentiation, or synergystic effect, is justified only if the combined effect far exceeds the sum of the separate effects. Thus if *E. coli* is retarded by 1,000 p.p.m. sulfaguanidine, and completely inhibited when, in addition, 100 p.p.m. of Triton K 12 are added (61) this result can hardly be called potentiation. But when *E. typhi* is only slightly retarded by 1,000 p.p.m. sulfaguanidine, and not at all by 100 p.p.m. Triton K 12, but is completely inhibited by 100 p.p.m. Triton+10 p.p.m. sulfaguanidine (61) that can be called potentiation (69). Since the effect of quats is inhibited by so many different factors (see above) it seems possible that potentiation may be accomplished by chemicals which remove some of the interfering or blocking compounds.

Appraisal of Bactericidal Efficiency

The customary evaluation of disinfectants by their phenol coefficients (87, 93, 94, 95) has proved unsatisfactory with quats (30, 39, 71, 72, 74 to 77). Different broths give different results (30, 71). Death time determinations are likely to give inconsistent results (55, 72). Cultures seemingly dead after five or ten minutes exposure may grow in the fifteen-minute transfer. Special precautions must be taken to prevent bacteriostasis by the disinfectant carried over with the transferred bacteria (85).

The bactericidal properties of quats are due to their chemical

reactivity and also to their great adsorbability. These same properties account also for their frequent failure. They are completely inactivated by soap (5), and by lecithin and other phospholipids (32, 33, 73); they are completely adsorbed by charcoal or bentonite (31, 50), also to a lesser degree by agar (51, 79, 83). They combine readily with proteins (5, 36, 91) and therefore are less efficient in presence of serum (55, 60) or milk (53, 54). Even some phosphates (103) appear to exert an inhibitory effect.

Another unusual feature makes the appraisal difficult. The logarithmic survivor curve is straight only for the killing of the first 99 to 99.9 per cent (77, 78). After that, the death rate decreases very noticeably, and the last survivors often display a surprising resistance (40). This has been explained, at least partially, by the agglutination of bacteria by quats (39). The bacteria inside the clumps remain alive longer. The clumps settle on the walls of the vessel and cause uneven sampling. To overcome this, Klarmann and Wright recommended using only 0.05 ml. of culture treating it with 0.5 ml. germicide, and after five, ten and fifteen minutes, adding 20 ml. of broth to the tubes, thus including survivors from clumps.

Another method developed by the same authors consists of using standard pieces of filter paper to which 0.5 ml. of culture and 5 ml. of disinfectant is added. After five, ten and fifteen minutes, one piece of filter paper is removed to 20 ml. of broth, and shortly afterwards transferred again to new broth to eliminate bacteriostasis. Both methods are far more severe than the phenol coefficient method (31).

In the standard phenol coefficient test, bacteria may retain for quite a while the film of adsorbed quats when transferred to a new medium, so that they cannot multiply and may be considered dead, unless this film is destroyed by soap, lecithin, or adsorbent charcoal. The addition of such compounds to the test medium has been recommended (28, 33, 79, 85). As no antidotes are used in assaying phenols, mercurials or sulfa drugs, it must be admitted that the use of antidotes only in the case of quats is quite arbitrary. A similar use of antidotes with phenols would show them to be less efficient, and it would indicate mercurials to be utterly useless. Mallmann (80) states "By selecting the proper laboratory technic, we can show that the cationics are either poor or unusually good disinfectants" Nevertheless, even the manufactures of quats

have recommended the use of antidotes to prove the efficiency of their products.

All these reactions greatly affect the usefulness of quats in the various applications, and for any specific case, the method for testing the germicidal efficiency should consider the kind or degree of efficiency that is needed. Absolute sterility, including the killing of spores, would be expected with surgical instruments, but in the cleansing of machinery and equipment for the manufacture of frozen foods, elimination of 99 per cent of the bacteria may be satisfactory. In still other cases, it may be immaterial whether the bacteria are killed or not, as long as they are prevented from multiplying. It is quite important in these latter cases to remember that a few cells or clumps may prove to be much more resistant than the large majority.

In order to get a clear picture of the efficacy of a disinfectant, the phenol coefficient, as an all-or-nothing method, must be substituted by a procedure which gives information concerning the percentage of survivors. Such a method has been introduced by Mallmann (81) in his "use-dilution method". The test bacteria are dried on glass rods which are dropped into the disinfectant solution. After various exposure times, the rods are removed, shaken in broth, and the survivors counted by agar plating. This technique has been found satisfactory for sanitation purposes.

The agar cup method has been shown to give results which do not agree with the phenol coefficient method (51, 83).

In vivo tests have been made to prove the efficiency of quats for the sterilization of skin and mucous membranes, and to justify their application as skin disinfectants, mouth washes, nose drops, vaginal suppositories, etc. For this purpose, Green *et al.* (82, 83, 84) used the chorioallantoic membrane of the chick embryo; Whitehill (89) used the rabbit eye; Kenner *et al.* (88) injected cultures plus quats into mice; Nungester & Kempf (90) and also Spaulding & Bondi (92) applied the "infection prevention method;" Herrell (96) used tissue cultures.

APPLICATIONS OF THE QUATERNARY AMMONIUM COMPOUNDS

General sanitation.—Krog & Marshall (98), using a swab test technique, established the bactericidal action of Zephiran against bacteria commonly found on eating and drinking utensils. A one-

minute exposure to a 1:5,000 dilution was apparently sufficient to reduce bacteria surviving on tumblers to less than 100 per rim. Subsequent studies (80, 99 to 102) have confirmed these results for this and other quats. The reaction of quats with soap or with phosphates used in cleaning compounds (103) does not seem to lower their efficiency when properly used in 2- or 3-compartment washing procedures. The quats have an advantage in being relatively odorless and tasteless in recommended concentrations, and have been accepted as a sanitizing rinse after washing by more than one hundred and sixty cities in eighteen states throughout the country (102).

Recently there has been considerable interest in the possibility of developing a dishwashing compound which would be germicidal as well as having good detergent properties, and which would thus combine two operations into one. Quats have been suggested for this purpose, but they are incompatible with the best detergents which are anionic. Progress has recently been reported by Guiteras & Shapiro (104) who used a nonionic alkylated aryl poly-ether alcohol as the wetting agent and cetyl-dimethyl-ethyl-ammonium bromide as the germicidal agent.

Quats have been suggested for use on the farm in sterilizing milking machine cups, etc., (105) and have been found effective in reducing the bacterial count from washed and supposedly clean milk cans when one ounce of 10 per cent solution was used in one gallon of water (106, 151). Scales & Kemp (105) and Frayer (107) considered Zephiran or Roccal useful for sterilizing cows' udders prior to milking. Recently Hughes & Edwards (108) in England reported lesions developing on cows teats apparently as the result of repeated application of another quat, CTAB.

Krog & Marshall (109) made a study of the use of Roccal in a milk pasteurizing plant and reported that the use of a 1:5,000 to 1:8,000 dilution of the compound following the usual cleaning treatment resulted in 68 to 99 per cent reduction in bacterial counts obtained from swabbings made at specified stations throughout the plant. Several workers (78, 109, 113) have reported that the quats they studied are not harmful to those metals commonly used in the fabrication of dairy and food processing equipment. Krog (110) has suggested the use of 1:5,000 aqueous Roccal on wiping cloths for the sterilization of "novelty equipment" in the ice cream industry. Directions for the application of the quats in

the dairy plant have been given by Vignolo (111) and Levowitz (112). An important point to be emphasized is that these compounds become less efficient in the presence of milk and hence can be used only to sterilize equipment which has been washed free of milk (53). Health authorities have hesitated to permit quats as general disinfectants in milk plants because they might be used to cover up high bacterial counts in milk. This does not seem justified, however, because it has been shown (54) that Roccal does not influence the bacterial count of raw or pasteurized milk at concentrations as high as 1:500, and hence could not be used by unscrupulous dealers to cover up unsanitary practices. Quats have been accepted for dairy purposes in about thirty cities in the United States (102).

The success quats have enjoyed in the sanitation of dairy plant equipment has led to the suggestion that they be used in other food industries. Lehn & Vignolo (113) suggested that a quat in a dilution of one ounce of 10 per cent solution to four gallons of water could be used in breweries to sterilize lauter tubs, brew kettles, hop strainers, fermenting tanks, and surface coolers by spray application, and to sterilize plate coolers, beer and wort lines, fittings, and pumps by circulating the solution through the various pieces of equipment. Similar application has been suggested for the sterilization of lug boxes and various pieces of equipment in the food processing industry (114). In these nontechnical papers, no data as to the efficiency of the quats under the proposed conditions are given, but it seems likely that they should be as effective here as in the dairy plant, provided their use is restricted to previously cleaned equipment. Penniston & Hedrick (115) considered a mixture of long-chain pyridinium chlorides useful in preventing contamination of the shells of eggs intended for dehydration. The usual count of three to six million microorganisms found on eggs after washing in water could be reduced to a few thousand or less by a three to five minute rinse.

Water supply disenfection.—Fair *et al.* (116) studied the action of ten quats against *Entamoeba histolytica* cysts and found that with some quats the cysts could be killed by 30 p.p.m. in ten minutes or by 10 p.p.m. in two hours. The required concentration increased with increasing density of cysts to be killed, but suspended organic matter and pH seemed to have only slight effects. At

least four of the compounds were effective cysticides: Fixanol, Nopco QCL, Ceepryn, and Sapamine K_w. Zephiran and Triton K60 may also be effective, but the data on these compounds are not complete. The authors suggest their possible usefulness in water disinfection, sanitation of eating utensils, disinfection of shellfish, cleansing and disinfection of contaminated vegetables and fruits intended for raw consumption, cleansing and disinfection of water mains, cleansing of water filters, and control of *Psychoda* in trickling filters.

Recently (117) Polymine-D, when used in solid form combined with mild alkalies, has proved effective for sterilizing two jute-packed water mains that had proved refractive to chlorine sterilization. For the larger main (3,000 ft.) one 7-day application of the germicidal solution was effective.

Disinfection in surgery.—Domagk (5) mentioned the sterilizing effect of Zephirol on skin, and suggested its use in surgery to disinfect hands before putting on rubber gloves. Since that time, a number of workers have reported various quats to be effective for sterilizing surgeon's hands in gloveless surgery, or for securing a sterile operative field (118 to 127, 149). However, Miller *et al.* (52) emphasize certain limitations of using quats for skin disinfection. Quats, specifically Zephiran and Phemerol, deposit an invisible, nonperceptible film on the hands, and this film retains bacteria underneath it and is very resistant to mechanical trauma. The inner surface of the film has hardly any bactericidal power, whereas the outer surface exerts a strong germicidal action. The formation of such a film has been explained by Rahn (50) as due to an oriented adsorption of the molecules of the detergent on an organophilic surface with the harmless organic ends directed towards the skin and the germicidal—NOH groups toward the outside. The outside of such a film remains sterile for some time, but beneath the film the bacteria on the skin remain alive.

Whenever swabbing techniques (122, 123, 124) or contact plate methods (121, 127) have been used to determine the efficiency of skin disinfection ,only the outside of this film was touched and the results are of course good. Much better are the skin-sectioning techniques (118, 119, 126), but these too are not entirely reliable for no antidote was used, and this is essential when testing quaternary ammonium compounds (28). In surgery, quats could

not be expected to exert a bactericidal action in incisions where they would come in contact with various types of organic matter, phospholipids, etc., which would act as antidotes. It is possibly significant however that quats on the surgeon's hands and on the operative field have resulted in no infection, or in less infection than with iodine tincture (52, 119, 121). Kramer & Sedwitz (125) reported that 1:200 Ceepryn tincture had been used routinely in operative cases at the Youngstown, Ohio Hospital for three years (about 18,000 cases) with no observed irritation and no single instance of infection. Sterilization of the surgeon's hands in gloveless surgery depends upon the stability of the film of adsorbed germicide and upon the possibility of its neutralization by contact with tissue fluids and blood. Sterilization of the incision itself must be considered doubtful.

It has also been suggested that solutions of quats may be used to sterilize bowls, baths, and surgical instruments (118, 123, 124, 128). More information is needed with respect to their use on surgical instruments. In Germany, Brekenfeld (129, 130) and Kayser (131) recommended boiling surgical instruments in army field hospitals in 0.75 to 2.0 per cent Zephirol for fifteen minutes, while Zeissler & Günther (132, 133) were against using Zephirol on the ground that it would not kill spores from soil.

Human medicine.—Quats have been used in attempts to combat various infections. They also have been found to be fungicidal (150). Wright & Wilkinson (120) considered 1:1,000 Zephiran effective in preventing infection in injuries and specifically mention burns. On the other hand, Barnes (123) found no sterilizing action of CTAB on burns. Forman (128) reported 1 per cent CTAB appeared effective in curing boils and dermatitis due to strapping and postulated the theory that it reduced the staphylococcal population of the skin so that natural bactericidal powers of the skin were sufficient to cope with the infection. Although one case appeared to show sensitivity, Forman found satisfactory progress in most cases in treating severe nummular eczema, chronic dermatitis , and impetigo of the face. Fischer (134) also used a quat in the prevention of impetigo in the newborn. Neter (135) showed that Zephiran 1:2,000 would inactivate tetanus toxin almost completely, which suggested its use in the prevention or treatment of infection by toxin-producing bacteria. But as Neter himself

pointed out, the addition of human serum to the Zephiran solution interfered with the toxin-destroying properties. Neter (136) has also shown that 1:10,000 Zephiran inhibits clotting of plasma by staphylococci, and 1:100,000 inhibits fibrinolysis by hemolytic streptococci. Barber (137) reported some success with 1:200 cetylpyridinium chloride tincture in preventing experimental *Streptococcus* infections in mice. The same compound was used successfully in bronchial lavage (152).

Although quats have been reported to be highly virucidal *in vitro*, against various strains of bacteriophages and viruses (138, 139, 153), Klein & Stevens (138) could not prevent experimental influenza virus infections in mice when quats were administered by intranasal spray. Other workers (140) have reported that Zephiran 1:10,000 will not inactivate type B influenza virus in one hour at room temperature. In fact quats have been used to preserve virus and bacteriophage suspensions (141, 142, 143).

Zephiran has been found effective in inhibiting the metabolic activities of microörganisms associated with dental caries (144) and there is some evidence that this compound (145) and cetylpyridinium chloride (146) are effective mouth antiseptics.

Veterinary medicine.—On the basis of *in vitro* tests against mastitis organisms Scales & Kemp (105) suggested that a mixture of Zephiran, a dispersant (Triton 720) and 33 per cent milk might be effective as a treatment for chronic mastitis if injected into the udder at body temperature directly after milking. Bryan *et al.* (147) have subsequently reported success in treating chronic mastitis by infusing each quarter with 75 ml. of 1:1,000 aqueous Phemerol.

Bryan & Young (148) have also reported success in curing ringworm of calves by twice weekly applications of 1:1,000 aqueous Phemerol to the scabs.

It is evident that caution must be exercised in the application of quats for Hughes & Edwards (108) found lesions developing on cows' teats apparently as the result of continued application of CTAB. While 1 per cent CTAB cream appeared effective in preventing the spread of *Streptococcus agalactiae* mastitis when applied twice daily to milkers' hands and cows' teats, after three months lesions of the teats developed accompanied by a rapid increase in *S. agalactiae* infection of teats and milk. 1 per cent

CTAB cream failed to control this infection, and it was suggested that the increase in the number of cows with teat lesions was due to the use of this compound.

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ANTIBIOTICS

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Introduction

The phenomenon of antagonism in mixed bacterial cultures has been studied for many years and has been explained in a variety of ways. The antagonistic effects of one species upon another have been ascribed usually to production of acid, to harmful enzyme systems, or to the production of hydrogen peroxide by the inhibiting organism. Recognition of the possibility of the production of specific, more or less complex, toxic materials was slow in forming, but such an explanation was in gradual development until penicillin appeared upon the scene. The development of such therapeutically useful substances as tyrothricin and penicillin led, not only to the present concept of antibiotics, but also to a flood of work in this field.

The purpose of this review is to report only the most recent developments in the study of antibiotics and no reference will be made to historical background. For historical as well as for recent information the reviews of Waksman (1), Oxford (2), and Frieden (3) should be consulted.

The term "antibiotic" as used in the sciences is not yet of fixed definition; it connotes many different meanings, depending on individual interests. For this discussion we choose to define an antibiotic as a chemical compound derived from or produced by living organisms, which is capable, in small concentration, of inhibiting the life processes of microorganisms. The reader will note that in this review no reference is made to the glucose oxidase of Müller, which has been variously designated as notatin, penatin, penicillin B, and *Escherichia coli* factor. We hold that because this enzyme owes its inhibitory character to hydrogen peroxide formation it is in fact not an antibiotic. On the other hand, we have included reference to many ill-defined substances which may be shown on closer inspection to owe their properties to secondary characteristics such as those displayed by glucose oxidase. Be-

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cause no recent information has appeared, the following antibiotic substances are not treated in this review: toxoflavin, micromonosporin, fumigatin, puberulic and puberulonic acids, spinulosin and gentisyl alcohol.

The organization of the text is based on the botanical classification of the organisms responsible for antibiotic production. Those substances produced by the lowest forms in the division *Thallophyta* are first considered, and, in turn, antibiotic products of progressively higher forms are discussed.

Antibiotics From Bacteria

Bacillus subtilis *group.*—More antibiotic entities have been described as products of strains of *Bacillus subtilis* than of any other species. Antibiotic activity is produced in a wide variety of media, including both inorganic and organic types.

SUBTILIN, first described by Jansen & Hirschmann (4) shows some evidence of polypeptidic nature (5) and is probably a complex (6) of several factors. It has been studied by Salle & Jann (7, 8, 9) who have shown it to be antagonistic chiefly to gram-positive bacteria. Acid-fast organisms, including *Mycobacterium tuberculosis*, and a number of pathogenic higher fungi are also susceptible. A remarkably low toxicity combined with marked *in vivo* protection against pneumococcus type III and *Bacillus anthracis* infections in guinea pigs (10) indicate that subtilin may become useful as a therapeutic drug. The same investigators (11) treated mice with subtilin nine hours after inoculation with a virulent strain of *Streptococcus hemolyticus*, and remarkable recoveries without apparent toxic reactions were observed. Anderson *et al.* (12) found subtilin to be active *in vitro* against *Endamoeba histolytica* and its associated bacterium "t," and also against *Trypanosoma equiperdum*. In these applications, it did not cause immediate hemolysis of erythrocytes.

BACITRACIN, first described by Johnson *et al.* (13) is produced by a member of the *B. subtilis* group, and resembles subtilin in a number of ways, but differs from the latter by accumulating primarily in the culture liquor free from the cells. It may be extracted readily with n-butanol, is not precipitable by pH manipulation and resists digestion with trypsin or pepsin. It is somewhat toxic but nonirritating to tissue, and has shown promise in mouse-protection studies against certain gram-positive pathogens. Local

treatment of one hundred cases of surgical infections (14) resulted in a favorable response in 88 per cent of the patients. Eagle *et al.* (15) have studied blood levels, urinary excretion, and renal clearance of nontoxic doses of bacitracin in rabbits and man. After intravenous and intramuscular injection, blood levels of 1 μg. per cc. were maintained approximately six times longer with bacitracin than with penicillin G similarly administered. Goorley (16) has determined certain chemical and physical properties of bacitracin and believes it to be a polypeptide of high molecular weight. It is soluble in water and the lower alcohols but insoluble in many other organic solvents. Pilot-plant operations have yielded material suitable for parenteral administration.

A third agent of *B. subtilis* has been named BACILLIN (anticoli factor) by Foster & Woodruff (17). This substance is readily produced on media containing carbohydrate. It may be distinguished from subtilin and bacitracin by its high activity against both gram-positive and gram-negative bacteria in certain media, its moderate toxicity for mice, and its complete ineffectiveness in protecting experimental animals from virulent bacterial infections. Blood or other complex natural materials in the medium reduces or eliminates the antibacterial properties of bacillin. The inactivating agent has been named antibacillin by Woodruff & Foster (18) and is thought to be a peptide, or a mixture of peptides.

The production of EUMYCIN by *B. subtilis* (Marburg strain) has been reported by Johnson & Burdon (19). This substance is without effect on gram-negative bacteria, inhibits staphylococci only slightly, and shows considerable activity *in vitro* against *Corynebacterium diphtheriae*, *Mycobacterium tuberculosis*, and some of the higher pathogenic fungi. It has low toxicity for mice. Later reports (20) show that some highly fungistatic eumycin fractions are hemolytic as well as toxic for mice. When the antagonist was grown in Youman's synthetic medium supplemented with manganous sulfate, filtrates were obtained which were less hemolytic and less toxic for mice and more active against acid-fast organisms than the highly fungistic fractions previously mentioned.

Callow & D'Arcy Hart (21) have recovered an antibiotic, subsequently termed LICHENIFORMIN, from the cells of *Bacillus licheniformis* Weigmann emend. Gibson. According to Smith (22) the responsible organism is probably identical with *Bacillus subtilis* (Ford strain). The active agent has little or no effect on

gram-negative organisms, but inhibits certain gram-positive and acid-fast microorganisms, including *Mycobacterium tuberculosis* and *M. phlei*. It does not resemble bacitracin, and was first differentiated from subtilin only on the basis of its insolubility in aqueous ethanol. On the basis of other studies the reviewers (6) were inclined to regard licheniformin as similar to, if not identical with, subtilin. However, recent information by Callow *et al.* (23) reveals that the antibiotic can be crystallized as the helianthate and retains high activity against acid-fast and many gram-positive bacteria. Mice of 20 gm. survived intravenous, intraperitoneal, or subcutaneous injections with 5.0 mg. of the material, while injections of 10 mg. were fatal. Mice experimentally infected with a licheniformin-sensitive *Staphylococcus pyogenes*, were partially protected by the antibiotic. In experimental anthrax, only 16 per cent of forty treated mice died, whereas 95 per cent of the controls succumbed. Some suppression of pulmonary tuberculosis in mice infected with bovine tuberculosis was effected by giving 6 mg. daily in divided doses for a period of forty days.

Several new factors from *B. subtilis* have been recently reported by various French workers. Among these are Vallée's (24) SUBTILYSIN (SUBTILYNE) which is strongly lytic towards certain gram-negative and gram-positive organisms, but is without effect on staphylococci or streptococci. Another, called ENDO-SUBTILYSIN by de Saint-Rat & Olivier (25) is reported to be nontoxic and, in high dilution, bactericidal for staphylococci. Ramon & Richou (26) have reported the formation of a substance called SUBTILINE which inactivates certain bacteria *in vitro* and which, *in vivo*, partially or completely overcomes the toxicity of tetanus, diphtheria, botulinus, perfringens, and staphylococcus toxins. Remlinger & Bailly (27, 28) have described an unnamed agent from culture filtrates which inactivated or destroyed strains of rabies virus and equine encephalomyelitis virus (Western type of the U.S.A.) *in vitro*.

Bacillus brevis *group*.—Considerable effort has been expended to determine the chemical nature of GRAMICIDIN and TYROCIDINE, the two components of TYROTHRICIN. According to Dubos (29), the latter contains approximately 20 to 25 per cent gramicidin and about 60 per cent tyrocidine. Gramicidin is pictured as a large cyclopeptide, approximately $C_{148}H_{210}O_{26}N_{30}$, mol. wt. 2826, with a high content of tryptophane together with natural

and unnatural isomers of other amino acids. The types and numbers of amino acid residues completely accounting for the nitrogen in a stoichiometric unit were deduced by Synge (30). Lewis *et al.* (31) treated gramicidin with formaldehyde which reduced its hemolytic activity to one-fifth and its antibacterial activity to one-half that of untreated gramicidin. Chemically, the reaction results in the introduction of methylol groups, probably on the 2-position of the indole rings of the tryptophane residues. Olcott *et al.* (32) found that esterification of the hydroxyl groups of methylol gramicidin with succinic anyhdride yields a product, the sodium salt of which is completely soluble in water, even in the presence of other ions. Unmodified gramicidin is so insoluble in aqueous media that its usefulness is greatly limited. The half-succinic acid ester derivative has approximately 25 per cent of the antibacterial activity, 1 to 5 per cent of the *in vitro* hemolytic activity, and only 2 per cent of the toxicity of gramicidin. Crystalline gramicidin is active only against gram-positive bacteria (*in vitro* and *in vivo*); activity *in vitro* is enhanced by the presence of one per cent serum albumen in the medium.

Tyrocidine hydrochloride is composed of numerous *l*-amino acid residues and three residues of *d*-phenylalanine. It has the approximate composition, $C_{127}H_{166}O_{26}N_{26}$-2HCl. mol. wt. 2564 (29). It is active against gram-positive and gram-negative bacteria only *in vitro*. Its loss of activity in animal tissues is probably due to inhibition by serum proteins. Gray & Kazin (33) found that tyrothricin added to brewers' yeast in concentrations up to 500 p.p.m. did not affect the growth or the fermenting power of the yeast nor did such treatment affect beer flavor. Gram-positive organisms, including species of *Sarcina*, *Lactobacillus pastorianus* and *L. delbreuckii*, were killed by tryothricin concentrations as low as 10 to 20 p.p.m.

The strain of *B. brevis* employed in the production of GRAMICIDIN S was first isolated in 1942 by Gause & Brazhnikova (34), and differs from Dubos' *B. brevis* in certain physiological characteristics. Crystalline gramicidin S, according to Synge (35) is a cyclopeptide hydrochloride having a stoichiometric minimum unit formed from one residue each of *l*-ornithine, *l*-valine, *l*-leucine, *l*-proline, and *d*-phenylalanine. Synge feels that this compound should be renamed, on the basis that it contains only one amino acid (*l*-valine) in common with gramicidin; and because it possesses

unlike gramicidin, considerable antibacterial activity against gram-negative organisms and appears to exert this effect by virtue of a different mechanism. Considerable success in the topical treatment of gunshot wounds in Russian soldiers has been reported by Gause (36).

Reitler & Boxer (37) have recently described a *B. brevis*-like isolate, from lymphatic glands of the human mesentery, which produced a lytic agent highly active against *Staphylococcus aureus*, α and β-hemolytic streptococci, pneumococci, *Pasteurella pestis* and *Paramecium caudatum*. This substance, although not yet obtained in crystalline form, has properties quite unlike those of tyrothricin.

From sporulating cells of *Bacillus larvae*, a bee pathogen, Holst (38) has obtained an agent which is active against gram-negative, gram-positive, and acid-fast organisms other than *Mycobacterium tuberculosis* var. *avian*. Purification is very difficult and the material is toxic on intraperitoneal injection into mice.

From culture filtrates of an unidentified yellow, aerobic, sporulating bacillus, Gause (39) has obtained a nontoxic bacteriostatic factor COLISTATIN, which strongly inhibits certain gram-negative pathogens and staphylococci, and has protected mice from large doses of *Borrelia sogdianum*, a relapsing fever spirochete. The properties ascribed to the active agent clearly differentiate it from tyrothricin, gramicidin S and bacitracin.

The complex chemical nature of antibiotics from spore bearing bacilli has made purification difficult. Chemical formulae have so far been offered only for gramicidin S and the components of tyrothricin. As more highly purified preparations are obtained and as the chemistry of these many factors is elucidated it is likely that a certain amount of duplication among factors will be disclosed. Oxford (2) has appropriately suggested that future workers in the field refrain from giving a natural product a definite name ending in "in" until it has been obtained in a reasonably pure state, and that they forego the use of such name if it becomes clear that another has previously purified and named the same compound.

Antibiotics from other bacteria.—In spite of all the information which has been accumulating for half a century, the antibiotic substances from *Pseudomonas aeruginosa* (*B. pyocyaneus*) have not been carefully studied until recently. Schoental (40) assisted in clarifying the situation by obtaining PYOCYANINE, α-HY-

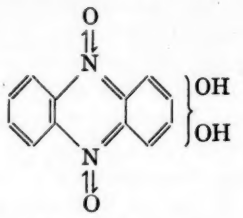
DROXYPHENAZINE and a third almost colorless compound from chloroform extracts of the organism. Hays *et al.* (41) made an exhaustive study of active agents recovered from 5-week cultures on nutrient glycerol broth. By hot-alcohol extraction of centrifuged cells, the following "Pyo" compounds were obtained: Pyo I, $C_{14}H_{10}N_2O_2$; Pyo II, $C_{34}H_{46}N_2O_4$; Pyo III, $C_{34}H_{44}N_2O_2$; Pyo IV, $C_{16}H_{23}NO_3$; Pyo 1b, $C_{31}H_{40}N_2O_2$; and Pyo 1c, $C_{34}H_{48}N_2O_2$. All of these substances have been crystallized with the exception of Pyo II. In general, they are active only against gram-positive organisms. Sufficient quantities of Pyo II have been prepared by Wells *et al.* (42) to permit study of additional biological properties of the compound. The lethal dose of Pyo II for mice is between 0.5 and 0.75 mg., and hemolysis of rabbit erythrocytes is not caused by concentrations up to 1.0 mg. per ml. Administration of Pyo II failed to protect mice against a virulent strain of *Diplococcus pneumoniae*, and the course of the disease produced by *Mycobacterium tuberculosis* (human type) in four guinea pigs was not altered by daily administration of this compound. Bergström *et al.* (43) have recently crystallized a substance (which they named PYOLIPIC ACID) from *P. aeruginosa* and which they found to be active against *M. tuberculosis*. Analysis of the substance provided the formula $C_{11}H_{22}O_3$ and the compound appears to be similar to, if not identical with, *dl*-β-hydroxydecanoic acid.

Lichstein & Van de Sand (44) have studied the antibacterial action of VIOLACEIN, the violet pigment of *Chromobacterium violaceum* and found marked inhibition *in vitro* against certain gram-positive bacteria but not against *Clostridium perfringens*. Mice were found to tolerate 2 mg. by intraperitoneal injection, but activity of the pigment was greatly reduced in the presence of serum. The same authors (45) obtained PRODIGIOSIN from three-week cultures of *Chromobacterium prodigiosum* (*Serratia marcescens*) and this thermostable, water-soluble, nonpigmented substance was active against *Bacillus subtilis*, *Corynebacterium diphtheriae*, and *Staphylococcus aureus*. PHTHIOCOL, a pigment from *Mycobacterium tuberculosis*, was active against a number of gram-positive and gram-negative bacteria.

A bacterial pigment closely related to α-hydroxyphenazine from *Pseudomonas aeruginosa* is IODININ (I) from *Chromobacterium iodinum*. This substance is a di-N-oxide of a dihydroxyphenazine and the position of the hydroxyl groups is still in doubt

(2). Weidling (46) found that iodinin was bactericidal against staphylococci and streptococci, bacteriostatic against *Eberthella typhosa* and weakly active against *Proteus vulgaris*.

From two strains of milk streptococci, Oxford (47) obtained DIPLOCOCCIN, an active protein-like substance of relatively small molecular size. It is active against other lactic cocci. Partial purification of this substance from cells grown on a synthetic medium (48) show that it contains no sulfur or phosphorus. It is



I

stable at pH 4.0 and 100° C. but not at pH 6 to 7. Whether diplococcin and an unnamed factor from group N streptococci described by Mattick & Hirsch (49) are alike cannot be determined on the basis of comparative data now available. The unnamed factor proved highly active against certain species of *Bacillus*, *Clostridium*, *Lactobacillus*, and *Streptococcus*, but was without effect against staphylococci and gram-negative bacteria. Crude preparations were well tolerated by mice on subcutaneous and intravenous injections, and complete protection against 10,000 M.L.D. of hemolytic streptococci in mice was afforded by 10 mg. of the material. These same workers (50) inoculated sterile milk with group N streptococci and human tuberculosis organisms simultaneously; tuberculosis did not develop when centrifugates of resulting two-day old cultures were used to inoculate guinea pigs.

Thompson & Shibuya (51) have demonstrated that the inhibitory action of saliva on the diphtheria organism is due to the presence of *Streptococcus mitis* (viridans group). They succeeded in developing the above agent in culture, and found its production to be greatly increased by the addition of small amounts of tryptose (0.2 to 0.5 per cent). In the presence of 2 per cent of tryptose, however, practically no antibiotic was formed.

Actinomycetes and Related Forms

Many actinomycetes and related forms from nature possess marked antibacterial properties. Burkholder (52) studied 7,369 cultures from soil and found that 1,869 (25 per cent) inhibited *Staphylococcus aureus*, 261 (3 per cent) inhibited *Escherichia coli*, and 514 (6 per cent) inhibited *Candida albicans*.

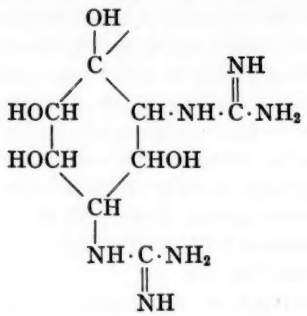
ACTINOMYCIN, the highly toxic antibiotic from *Actinomyces antibioticus*, was formerly considered a complex of actinomycin A and actinomycin B. However, it is now thought by Waksman *et al.* (53) to be a single entity since the actinomycin B fraction is never obtained in large yields, and there are indications that the activity of this fraction is caused by low concentrations of the same substance which occurs in the A fraction. These authors have examined some 10,000 cultures of actinomycetes since 1940 and have found only two additional actinomycin-producing strains.

According to Fainshmidt & Koreniako (54), MYCETIN, an antibacterial substance elaborated by *Actinomyces violaceus*, differs in certain respects from actinomycin and actinomycetin. Partially purified mycetin, obtained by extraction of agar on which the organism had grown, is deep violet in color and highly active against *Staphylococcus aureus*. Other active agents from *Actinomyces violaceus* include ACTINOMYCES LYSOZYME (55) and ACTINOMYCETIN (56, 57).

STREPTOTHRICIN, an active agent from *Actinomyces lavendulae*, has been purified in the form of its crystalline reineckate by Fried & Wintersteiner (58). Little is known of its chemical nature except that the crystalline reineckate contains 20 to 22 per cent nitrogen, has no methoxy, N-methyl or hydrolyzable acetyl groups. Peck *et al.* (59) succeeded in preparing streptothricin hydrochloride of high purity which showed an activity of 830 units per mg. based on a cup assay method with *Bacillus subtilis*. In addition to its marked effect on both gram-negative and gram-positive bacteria, streptothricin has been shown by Reilly *et al.* (60) to have powerful fungistatic and fungicidal properties against some of the higher pathogenic fungi. Among the numerous toxicity studies made on this drug are those of Rake *et al.* (61) and Molitor (62). With mice, intravenous administration of the drug recovered from certain media results in two toxic phenomena: an immediate reaction due to an impurity with histamine-like activity, and a delayed reaction

due to streptothricin itself which leads to death in two to twelve days.

No single antibiotic since the advent of penicillin has created as much interest as STREPTOMYCIN. A tremendous amount of work has been done in determining the chemical nature of this complex compound by chemists in a number of research laboratories, including those of E. R. Squibb and Sons, Merck and Company, The University of Illinois, Ohio State University, and Abbott Laboratories. Crystalline streptomycin has the probable formula $C_{21}H_{39}N_7O_{12}$, (II, III, IV) and is compounded of the following entities: Streptidine, (II) a meso form of 1,3-diguanido-2,4,5,6-tetrahydroxycyclohexane; streptobiosamine, consisting of N-methyl-*l*-glucosamine (III) and the streptose moiety (IV).



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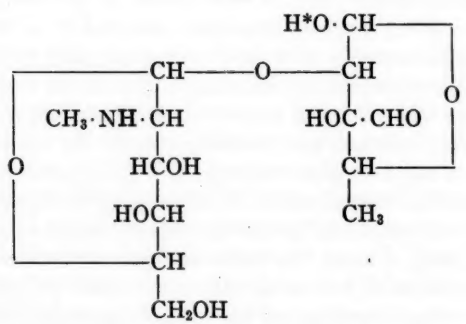


* Point of attachment of streptidine moiety (II)

III IV

The structures of streptidine (II) and of N-methyl-*l*-glucosamine (III) have been proven by the preparation of many derivatives. The molecular arrangement for streptobiosamine (IV) shown above is one provisionally suggested by Kuehl *et al.* (63). It is included in this review only to show how N-methyl-*l*-glucosamine may be linked to the streptose moiety. Fried & Wintersteiner (64) have shown that streptomycin possesses an aldehyde group which resides in the maltol forming moiety, $C_6H_{8-10}O_5$. They further point out that there is a tendency of some dicarbonyl sugars to undergo rearrangement in alkaline media. Thus, the position of the carbonyl groups, the points of attachment on streptidine and of N-methyl-*l*-glucosamine, and the functions of the remaining oxygen groups are still unknown.

Streptomycin has a remarkably low toxicity for man and lower animals as shown by Molitor (62) and Robinson (65) in their reviews on streptothricin and streptomycin. Unfavorable reactions due to a histamine-like compound associated with the latter have been eliminated in commercial production by radical changes in the medium in which *Streptomyces griseus* is grown. Space is not available to discuss the clinical use of streptomycin in the treatment of various diseases; the reader is referred to an excellent review on this subject by Keefer *et al.* (66) wherein treatment of 1,000 cases is reported.

Waksman *et al.* (67) have reported a second antibiotic from *Streptomyces griseus*, distinct from streptomycin on the basis of solubility in organic solvents and its lack of effect on gram-negative bacteria. This factor is now called GRISEIN (68).

Existence of a third factor from this organism seems likely from the work of Whiffen *et al.* (69) who have reported an antifungal agent highly active against *Cryptococcus neoformans* and certain yeasts. This agent resembles grisein in its ether solubility, but differs markedly in its action against *B. subtilis*. Grisein inhibits *B. subtilis in vitro* at 1:800,000 dilution, whereas the agent of Whiffen and co-workers has no effect on this organism at 1:10,000 dilution.

PROACTINOMYCIN, contained in extracts of culture fluids on which *Nocardia gardneri* has been grown, resembles penicillin in action but is more stable and more toxic than the latter. Florey *et al.* (70) in a pharmacological study of its effect on experimental animals found that if given by mouth, proactinomycin protects mice against intraperitoneal infection by hemolytic streptococci,

although the blood shows no antibacterial effect even after large doses. They suggest that it may be useful clinically where other less toxic antibiotics are not effective.

From an organism identified as *Nocardia coeliaca*, Emmart (71) has obtained a substance named NOCARDINE, which has tuberculostatic activity against slow growing strains of *Mycobacterium tuberculosis*, human and bovine. Partially purified nocardine mixed with virulent tubercle bacilli and inoculated on chorioallantoic membranes of the chick embryo, produced fewer tubercles per membrane than the control suspensions. Guinea pigs inoculated with part of the same suspensions were not protected from infections but maintained much higher weights than the controls.

An antibacterial pigment produced by *Proactinomyces cyaneus antibioticus* has been described by Gause (72) and its chemistry studied by Brazhnikova (73). This substance, an anthocyanidine derivative termed LITMOCIDIN, was shown to have strong bacteriostatic action *in vitro* against staphylococci, streptococci, *Vibrio comma* and the tubercle bacillus. Although its action was not affected by serum *in vitro*, it gave no *in vivo* protection in mice inoculated with staphylococci very susceptible to its action *in vitro*. It occurs in two forms: acid (red) and alkaline (blue) possessing different solubilities in water. Both forms inhibit *Staphylococcus aureus* in a dilution of 1:4,000,000. The pH at which both forms were tested against *S. aureus* was not given. Thus some of the inhibition of this organism may have been due to an unfavorable reaction of the medium.

From cultures of antagonists intermediate between *Streptomyces lavendulae* and *S. reticulus-ruber*, Woodruff & Foster (74) have obtained an agent which they call STREPTIN. Concentrates of this agent are more effective against staphylococci and micrococci than are comparable preparations of streptothricin and streptomycin. Differentiation of streptin was made mainly on the basis of its greater effectiveness against staphylococci and micrococci. More convincing evidence would be furnished by the ability of streptin to inhibit streptomycin- and streptothricin-fast strains of the test organisms.

Antibiotics From Yeasts

There are few reports in the literature of antibiotic agents isolated from yeasts. Carpenter (75) found that cultures of *Torula*

(*Torulopsis*) *utilis* var. *thermophila* and *T. utilis* var. *major* would inhibit the growth of *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Escherichia coli*, *in vitro*. Since the pH of the medium was reduced to 5.0 or lower, much of the inhibition may have been due to the formation of acid by the yeasts. It was further reported that topical applications of a dynamic culture of *T. utilis* var. *major* proved of value in several cases of secondary infection, after primary infection with *S. aureus* had been eliminated with topical applications of culture solutions of *Penicillium notatum*.

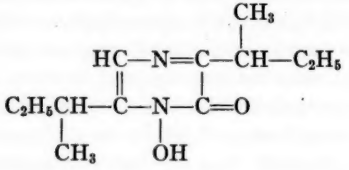
Takahashi (76) has made further studies on the chemical nature of a tobacco mosaic virus inactivator, obtained from bakers' or brewers' yeast, which he first reported in 1942. The substance is believed to be a polysaccharide since it yields osazones indistinguishable from glucosazone. Further tests showed that the substance was not a glucoside and was electrophoretically homogeneous as indicated by a single boundary in the Tiselius electrophoresis cell. Electron micrographs of inactivated virus showed no detectable evidence of gross change or disintegration.

Antibiotics From Molds

Extensive misuse of the term ASPERGILLIN as applied to certain antibiotic agents has finally been pointed out by Tobie (77). This term was originally used by Linossier in 1891 to designate the black pigment from spores of *Aspergillus niger*. This pigment has subsequently been shown to be a humic acid. Bush & Goth (78) have withdrawn the name aspergillin formerly applied to an active agent from *Aspergillus flavus* and have replaced it with flavacin. Stanley (79) gave the name aspergillin to an antibiotic obtained from cultures of *Aspergillus fumigatus*. More detailed studies on this material have shown it to be similar to if not identical with gliotoxin (80). However, the name aspergillin is still used to describe a factor which Soltys (81) isolated from cultures of *A. fumigatus* N.C.T.C. No. 366. It is active against both pathogenic and saprophytic mycobacteria. Krassilnikov & Koreniako (82) have also applied this term to a substance produced by certain strains of *Aspergillus niger*. These investigators claim that aspergillin resembles penicillin in its bactericidal properties but is distinguished from the latter by its inhibition of gram-negative bacteria and by its greater stability. It is little affected by the presence of pus or blood serum and is nontoxic for animals. Its protective

activity for test animals *in vivo* was not given. If the antibiotics described by Soltys and by Krassilnikov and Koreniako prove different from any previously described, the reviewers feel that other designations should be applied, and that use of the term "aspergillin" for antibiotics should be discontinued in any case.

The structure of ASPERGILLIC ACID, an antibiotic from *Aspergillus flavus*, was elucidated by Dutcher & Wintersteiner (83). It is a monobasic acid, $C_{12}H_{20}O_2N_2$ (V) This compound is



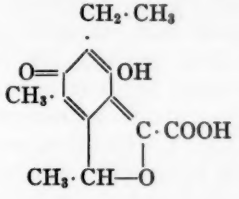
V

active against bovine, avian and human strains of *Mycobacterium tuberculosis* and a wide variety of both gram-positive and gram-negative bacteria. Extensive studies on the chemistry of compounds isolated from *A. flavus* culture liquors were made by Bush *et al.* (84). They isolated a pure aspergillic acid-like compound and obtained evidence that at least one other, somewhat less stable, antibiotic substance is present in crude preparations. Goth (85) found that iron interferes with the antibiotic action of aspergillic acid. He tried other metals (86) and obtained a great increase in the activity of aspergillic acid with cobalt, nickel, zinc, arsenic, and bismuth, the last-named being the most effective. Bismuth, in a concentration which in itself caused no growth inhibition, decreased considerably the amount of aspergillic acid needed to inhibit completely the multiplication of *Staphylococcus aureus*. The presence of human serum decreased the potentiating effect. Purified crystals from culture liquors of a strain of *Aspergillus flavus* were found by Salvin (87) to inhibit seven of eight pathogenic dermatophytes. Comparative studies indicate that this substance is similar to, but probably not identical with, aspergillic acid.

Interest in CHAETOMIN, an antibiotic from *Chaetomium cochlioides*, has not been maintained since it has been found that animals are not protected against bacteria susceptible to it *in vitro*.

Reilly *et al.* (88) found it to have practically no antifungal properties.

Although CITRININ ($C_{13}H_{14}O_5$) was originally reported in 1931, the structure (VI) proposed for it by Coyne *et al.* (89) is seriously challenged by Gore *et al.* (90) and by Sprenger & Ruoff (91). Gore and co-workers coupled diazotized *o*-chloraniline, 2:5 dichloraniline and sulfanilamide to citrinin to yield the correspond-



VI

ing mono-azo dyes. Coupling took place in the normal manner, in the *ortho* or *para* position with respect to the phenolic hydroxyl. The *ortho* and *para* positions shown in Coyne's formula above are already occupied and therefore could not be diazotized. The melting points of two synthetic derivatives of citrinin prepared by Sprenger and Ruoff were compared with corresponding derivatives of the natural material (89) and found to differ as much as 50° C. Citrinin exerts a selective action on gram-positive bacteria and is easily prepared in crystalline form, but its clinical use is limited because of its toxicity. Ambrose & De Eds (92) found doses of 50 mg. per kg. or higher to be toxic on parenteral administration to rats, rabbits, and guinea pigs. Chu (93) found that the sodium salt of citrinin showed very little irritation or other injurious effects when applied to the mucous membranes and skin of animals and man.

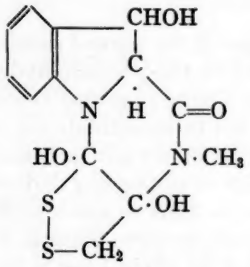
CLAVIFORMIN[1] (anhydro-3-hydroxymethylene-tetrahydro-γ-pyrone-2-carboxylic acid) is also known as clavacin, clavatin, patulin, and expansin. It also appears likely that penicidin, reported in a series of papers (1942–1944) by Atkinson and coworkers is the same as claviformin (95). It is readily obtained from

[1] Claviformin, the designation given preference by the reviewers, is the name applied by Florey & Jennings (94) who first crystallized the compound but did not determine its chemical structure.

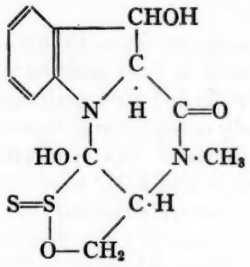
several species of *Penicillium* and *Aspergillus* as shown by Kent & Heatley (96) and from one species of *Gymnoascus*. Attempts to synthesize claviformin have been made by Attenburrow *et al.* (97) without success. Herrick (98) studied the antifungal properties of claviformin and found it highly active against *Trichophyton gypseum* but only slightly so against *Monilia albicans* and *Oidium asteroides*.

FUMIGACIN, $C_{32}H_{44}O_8$, an antibiotic obtained from *A. fumigatus* and *A. fumigatus* mut. *helvola*, is called helvolic acid by English workers in the field. Jennings (99) has found that it inhibits the growth of tubercle bacilli in addition to its previously recognized activity against various gram-positive bacteria.

Although GLIOTOXIN, $C_{13}H_{14}O_4N_2S_2$, was first crystallized in 1936, derivation of the structural formula has been difficult. Dutcher *et al.* (100) have suggested the formulae shown in (VII, VIII) as possible structures. The antibiotic is produced by various species of *Penicillium*, *Trichoderma*, and by *Aspergillus fumigatus* (80). Weindling's original gliotoxin producer, which he diagnosed as a *Trichoderma* sp., was later reexamined by Thom (101) and Timonim both of whom identified it as *Gliocladium fimbriatum*. Later, Brian (102) presented evidence to show that this strain is *Trichoderma viride*. Gliotoxin is active against many bacteria, actinomycetes and fungi, but its instability except at low pH levels limits its value as a fungicide for plant or animal infections (103). The origin of a toxicity to mycorrhiza in Wareham Heath soil was thought by Brian *et al.* (104) to be due to the accumulation of gliotoxin and other antibiotics produced by the Penicillia dominating its mold flora.

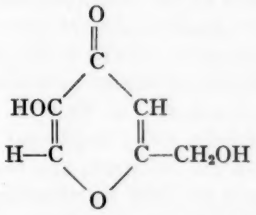


VII



VIII

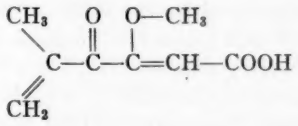
It is unfortunate that KOJIC ACID (IX) is not more effective against bacteria, and less toxic to laboratory animals and man



IX

since high yields of this compound may be readily obtained. Morton *et al.* (105) found kojic acid to be weakly active against a large number of gram-positive and gram-negative bacteria but active in high dilution against *Leptospira icterohemorrhagiae* and *L. canicola*. The M.L.D. for mice is about 30 mg. given intravenously.

PENICILLIC ACID, $C_8H_{10}O_4$, (X), a product derived from culture liquors of certain species of *Aspergillus* and *Penicillium*, is effective against both gram-negative and gram-positive bacteria but is toxic to mice in levels of 0.3 gm. per kg.



X

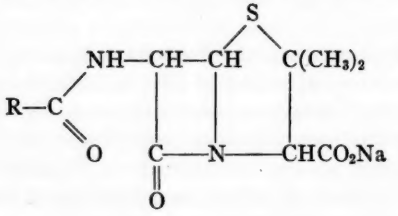
A tremendous amount of information has been obtained regarding the nature and action of PENICILLIN through the coordinated efforts of many workers in this country and abroad.

The commercial production of penicillin in the United States was made feasible by the development of improved media, improvement in methods of culture and selection of high producing strains. Production media based on corn steep liquor, mineral salts and lactose, and adaptation of the submerged method of culture were the contributions of Moyer & Coghill (106, 107). From hundreds of samples of moldy fruits, soils, and other materials, Raper and co-workers (108) isolated members of the *Penicillium notatum-*

chrysogenum group in a search for more productive penicillin producers. In combination, these efforts resulted in improving penicillin yields from 2 to 4 Oxford units per ml. to 160 to 220 units, and in establishing the practicability of commercial penicillin production. By irradiation of the conidia of Raper's highest producing strain, two greatly improved strains were obtained which in turn, found industrial application. The first of these, designated as X-1612, was isolated from x-ray irradiated conidia by Demerec and associates at Carnegie Institute, and the second, Q-176, was developed by Backus *et al.* (109) by ultraviolet irradiation of the X-1612 strain. With Q-176, concentrations of penicillin as high as 1,000 units per ml. of culture liquor may be obtained.

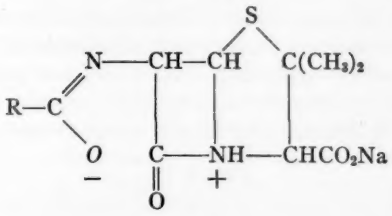
Penicillin in aqueous solution is extremely unstable in acid or alkali, and Benedict *et al.* (110) found that pH 6.0 is the apparent maximum stability point for aqueous solutions of crystalline penicillin G. The half-lives of crystalline penicillins were determined at pH 2.0 and 24° C., and were found to be 7, 11, 11, and 18.5 minutes for penicillins K, F, X, and G (111). The requirement for low temperatures during penicillin recovery is apparent.

The determination of the basal structure of the penicillin molecule proved to be an exceedingly difficult and time-consuming task. Brief summaries of the results of a cooperative program of chemical investigations sponsored by the Office of Scientific Research and Development (U.S.A.) and the Medical Research Council, Great Britain, have been published (112, 113). The sodium salts of two possible molecular configurations are given. The β-lactam structure (XI) is more favorably received than is the incipient



XI

azlactone version (XII). To illustrate chemical differences between various recognized penicillins and to show their comparative activities we have taken two tables from Rake & Richardson (114)



XII

and have revised them to include new data. Table I shows the types of natural penicillins described up to the present.

TABLE I

TYPES OF NATURAL PENICILLIN

Name	Synonym	Source	R—
F	I	*P. chrysogenum-notatum*	$CH_3CH_2CH{=}CHCH_2$—
Dihydro F	Gigantic acid	*A. giganteus*	$CH_3CH_2CH_2CH_2CH_2$—
Flavicidin, (115) Flavicin	F type	*A. flavus* *A. flavus*	$CH_3CH{=}CHCH_2CH_2$—
G	II	*P. chrysogenum-notatum*	
X	III	*P. chrysogenum-notatum*	
K	IV	*P. chrysogenum-notatum*	$CH_3CH_2CH_2CH_2CH_2CH_2CH_2$—
Abbott No. 128 (116)	?	?	?

Table II shows the activities of the various known penicillins in terms of the international unit (117) which is defined as the activity of 0.6 micrograms of crystalline penicillin G (the international standard) as measured against one of two specified strains of *Staphylococcus aureus*. Also given are the activities of the various penicillins again referred to the international standard but measured against a particular rough variant of *Bacillus subtilis* NRRL B-558. The ratios of activity are characteristic of the various types of penicillin and are useful in characterization or in estimation of

the nature of an unknown sample. A method developed by Schmidt *et al.* (118) based on this phenomenon of differential response was the first proposed for analysis of mixtures of two penicillins. By a modification of the Schmidt method employing three assay organisms, Higuchi & Peterson (119) can analyze mixtures of three

TABLE II

COMPARATIVE ACTIVITIES OF VARIOUS CRYSTALLINE PENICILLINS

Penicillin type	*Activity in units/mg. against*		*Ratio of activity*
	Staph. aureus	*B. subtilis* R.	vs. *B. subtilis*/ vs. *S. aureus*
G (II)	1667*	1667*	1.0*
F (I)	1465	970	0.65
Flavicidin	1400	1000	0.72
X (III)	850	1450	1.4 to 2.0
K (IV)	2300	760	0.33
Abbott No. 128	3500	—	—

* By definition.

penicillins of known types. Their method is more adaptable to routine laboratory practice than that of Goodall & Levi (120), who have described a modified partition chromatographic method with which relative amounts of penicillin I, II, III, and K may be determined, as well as new, yet undescribed types.

Because of evidence for its ineffectiveness and its instability in the body (116), penicillin manufacturers are interested in preparing a product containing as little of the K type as possible. Some doubt as to the rapid destruction of penicillin K in the body is expressed by Richardson (121) who believes that this entity is bound to blood plasma to a much greater degree than is penicillin G, and thus explains the low recoveries of K in the urine of animals injected with it. Moyer & Coghill (122) were among the first workers to recognize the value of using various precursors, including phenylacetic acid, to encourage the production of penicillin G. Higuchi *et al.* (123) have found that *Penicillium chrysogenum* Q-176, normally produces 87 to 88 per cent penicillin K. However, in the presence of β-phenylethylamine and phenylacetic acid there is a great increase in the proportion of G and an increase in unit yield. Although the first report of the isolation of synthetic peni-

cillin G appeared recently (124), the extremely low yields and tedious methods required indicate that the synthetic product will offer no competition to the biological product for some time to come.

Commercial penicillin producers are expending considerable effort to produce crystalline salts for the market. However, evidence has been accumulating during the past two years of the existence of an unnamed factor in amorphous penicillin which markedly enhances penicillin activity, though it lacks activity *per se*, *in vivo*. Welch *et al.* (125) have found that the factor is not inactivated by penicillinase nor by heating at 100° C. for forty-eight hours. It enhances the effectiveness of each of the fractions G, F, X, K, and dihydro F proportionately to their basic *in vivo* activity. Random samples of relatively old commercial lots of penicillin produced by fifteen different manufacturers indicate that approximately 60 per cent contain the factor. Single lots of amorphous penicillin of recent manufacture from eleven producers showed that about 40 per cent contain the factor.

Space does not permit discussion of the incalculable service rendered by penicillin in the treatment of various diseases. Several books on this subject have already been printed. Among other interesting features and uses for penicillin, De Ropp (126) has reported that penicillins G and X cause a reaction on the growth of excised fragments of sunflower stem tissue similar to that induced by indoleacetic acid. Both penicillin and streptomycin have been of value in reducing bacterial contamination in specimens used to inoculate eggs for the cultivation of certain viruses. Rose *et al.* (127) inoculated chick embryos with centrifuged human sputums after treatment with penicillin and streptomycin singly and in combination. The combination of agents proved to be more effective than either alone in preventing bacterial contamination and in favoring survival of the embryos. Morin & Turcotte (128) have found that penicillin in levels of 56 units per ml. added to smallpox vaccine emulsion had no effect on the virus but killed gram-positive cocci and prevented growth of spores of certain strict anaerobic pathogens. The latter were found to have lost their pathogenic power on subsequent isolation and testing. Thus, with this treatment, vaccine which previously had to be discarded could be used.

Some of the penicillin-like substances formerly referred to by other names (flavicidin and flavicin) have now been recognized as

F type penicillins. Several new species of *Penicillium*, producing penicillin-like antibiotics have been added to the growing list by Johns *et al.* (129). Chemical studies on these active agents leave little doubt that they are true penicillins. A penicillin-like compound has been reported from a pathogenic fungus (*Trycophyton mentagrophytes*) by Peck & Hewitt (130). Less well defined is the agent known as PENICILLIN-CRUSTOSIN obtained from strains of *P. crustosum* by Yermolieva *et al.* (131) who state that the clinical effect of their product corresponds generally with that of Fleming's penicillin.

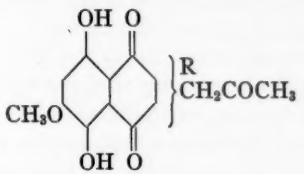
According to Ramon *et al.* (132) filtrates from cultures of *Penicillium notatum* possess, in contrast to penicillin, the ability to destroy such microbic toxins as staphylococcus, diphtheria and tetanus. This action, probably brought about by enzymes, is not destroyed by formaldehyde, heating at 55° C. for 30 min., or storage for three months. Human serum delays but does not inhibit the toxin-destroying effect. Miller & Boor (133) who found that penicillin protected mice against the lethal action of gonococcal endotoxin, have extended their studies (134) to other gram-negative endotoxin producers. The administration of penicillin to mice enabled them to survive significantly greater doses of endotoxins elaborated by a number of gram-negative bacteria, including two pathogenic *Neisseria*, members of the *Salmonella* group and *Shigella paradysenteriae*. A notable exception was the toxin of *Shigella dysenteriae* against which penicillin was ineffective.

In an unnamed factor, first reported from *Aspergillus ustus* by Kurung (135), activity is limited almost exclusively to certain acid-fast organisms including *Mycobacterium tuberculosis* and *M. ranae*. Using Kurung's culture, Hogeboom & Craig (136) employed the countercurrent distribution method to isolate two crystalline and one partially crystalline compound with high *in vitro* activity. The suggested empirical formulae were $C_{21}H_{17}Cl_3O_6$ and $C_{21}H_{18}Cl_2O_6$. The presence of halogen atoms show these to be unusual compounds. Further studies have been made by Doering *et al.* (137) on metabolic products of *A. ustus*. From culture filtrates three fractions were obtained and from one of these, a compound analyzing as $C_{19}H_{15}O_6Cl_3$ was named USTIN and considered similar if not identical to the $C_{21}H_{17}Cl_3O_6$ compound of Hogeboom and Craig. In synthetic media, compounds from the three fractions inhibit the growth of gram-positive cocci and mycobacteria. Ac-

tivity increases markedly with increase in pH but is sharply inhibited by the addition of serum albumin and certain other substances.

From the five-week old mycelium of *Penicillium puberulum*, which also produces puberulic and penicillic acids, Campbell *et al.* (138) isolated a photosensitive compound, $C_{17}H_{12}N_2O_2$. Two enolic hydroxyl groups are believed to be present in the molecule. Although antibiotic properties are claimed for the compound, information is lacking on the types of organisms affected.

From *Fusarium javanicum*, Arnstein *et al.* (139) have isolated two crystalline pigments which are highly active against *Staphylococcus aureus*, *Mycobacterium phlei*, and *M. tuberculosis* (human). The first of these, named JAVANICIN, analysed as $C_{15}H_{14}O_6$ (XIII) and the second, $C_{15}H_{14}O_7$; for javanicin, R equals CH_3, and for the second compound, R equals CH_2OH.



XIII

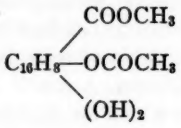
From *Penicillium gladiolii*, Brian *et al.* (140) have isolated a crystalline compound, $C_{11}H_{10}O_5$, which they named GLADIOLIC ACID. It inhibits the growth of certain gram-positive bacteria but has little or no effect on gram-negative bacteria.

In addition to producing gliotoxin, *Trichoderma viride* was found by Brian & McGowan (141) to produce a highly fungistatic substance for which the name VIRIDIN was suggested. This substance, unstable except at low pH, prevented the germination of the conidia of *Botrytis allii* at concentrations as low as 0.005 μg. per ml. Further study by Brian *et al.* (142) has led to crystallization of the compound (XIV) which is shown to have the empirical formula, $C_{20}H_{16}O_6$. They consider viridin unique in possessing negligible antibacterial properties. Loss of fungistatic properties at pH 3.5 is pronounced in solutions containing peptone, Marmite or a commercial yeast extract.

Asheshov & Strelitz (143) have found an active unnamed factor

from *A. fumigatus* which inhibits staphylococci and human tubercle bacilli but has no effect on avian tubercle bacilli.

A staphylocidal principle elaborated by *Penicillium corylophilum* Dx. has been extensively studied by Levaditi and co-workers (144). The active agent, named CORYLOPHILIN, is bactericidal for staphylococci in high dilution (10^{-9}) but has little



XIV

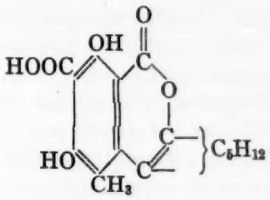
effect on pneumococci. *Escherichia coli* was inhibited at 10^{-3} and *Trypanosoma equiperdum* at 10^{-5} dilution. Blood serum inhibits its action on staphylococci, hence it has no beneficial action in rabbits infected with *Staphylococcus aureus*.

According to Ledingham (145) an antibiotic from *Penicillium claviforme*, MYCOIN C, was produced commercially by German interests during the war. Its solubilities in various organic solvents are similar to those of claviformin, but Mycoin C displays sensitivity to light and marked instability to heat, properties not possessed by claviformin. Extracts of the drug were incorporated in salves, powders, and jellies for external use.

Certain strains of *Penicillium janczewskii* Zal. were found by Brian *et al.* (146) to produce a "curling factor" which brings about an unusual stunting and distortion of the germ tubes and hyphae of *Botrytis allii* and other fungi. This effect is noted in concentrations as low as 1.0 μg. per ml. McGowan (147) has crystallized the curling factor, and chemical studies thus far indicate the empirical formula $C_{20}H_{20}O_9$, and that there are three methoxyl groups in each molecule. One methyl group is attached to carbon and no evidence for hydroxyl groups has yet been obtained.

Another fungistatic agent recently described is GLUTINOSIN, isolated from culture liquors of *Metarrhizium glutinosum* Pope by Brian & McGowan (148). The empirical formula of the crystalline compound is $C_{48}H_{60}O_{10}$. Produced with glutinosin is a biologically active material which causes a dermatitis similar to that produced by poison ivy. Therefore, it is essential to use barrier creams when working with culture extracts of the mold.

Culture liquors from *Penicillium brevi-compactum* were found by Abraham (149) to contain *NOR*-MYCOPHENOLIC acid (XV). He showed that although the compound inhibited *Staphylococcus aureus*, the organisms are easily adapted to growing in its



XV

presence. Further studies on this compound were made by Florey *et al.* (150) who found it to be fungicidal or fungistatic to a number of human fungal pathogens besides being partially inhibitory to the growth of various plant pathogens.

From an unidentified *Aspergillus*, Gerber & Gross (151) have found an agent which is both bacteriostatic and bactericidal for human tubercle bacilli *in vitro*. The substance has been named MYCOCIDIN.

Rosenthal (152) has reported from a mold, a red pigment which is active against the paratyphoid-typhoid-dysentery organisms but not against gram-positive organisms. Identification of the mold was not possible because fruiting structures failed to develop. Purification was effected by a solvent extraction method used for penicillin recovery. Upon intravenous injection of the purified substance into a rabbit, the activity was rapidly excreted in the urine.

In a search for fungi capable of inhibiting the growth of human tubercle bacilli, Boissevain (153) found that a strain of *Fusarium scirpi* produced a heat-stable agent which was active at low dilution against the test strain.

Antibiotic Activity of Phycomycetes

The only inhibitory agent yet described as the product of a phycomycete is one reported by Schatz *et al.* (154). The agent, produced by a *Phytophthora* or *Pythium* sp., inhibits the growth of *Trypanosoma equiperdum* but has little or no effect on *Escherichia*

coli, *Bacillus subtilis* and *Sarcina lutea*. No protective action was displayed when mice infected with the trypanosome were treated with crude preparations of the antibiotic.

Antibiotics From Basidiomycetes

The Basidiomycetes comprise some 30,000 species, including the shelf fungi, puff balls, toadstools, mushrooms, etc. Robbins *et al.* (155) have examined 300 species, most of which were included in the genera *Polyporus*, *Fomes*, and *Poria*, and of these 89 caused inhibition which was not due to the acidity of the culture liquors tested. *Escherichia coli* and *Staphylococcus aureus* were routinely employed as assay organisms, and culture fluids were tested periodically during a thirty-day incubation period. Bose (156) has suggested that basidiomycete culture liquors should be tested after incubation periods longer than thirty days and that test organisms other than *S. aureus* and *E. coli* should be used. In his experience, some basidiomycete culture liquors were active at three months and had shown periodic rises and falls in titer. Bose (157) also found that extracts of *Polystictus sanguineus* yielded a thermostable substance, POLYPORIN, which has high activity against many gram-negative and gram-positive bacteria. Little is known of the chemical nature of the substance except that it is acidic and that the sodium salt retains activity for long periods. It was found to be nontoxic for guinea pigs and rabbits and effective in protecting the former against lethal doses of *Vibrio cholerae* and *Eberthella typhosa*. Clinical trials of crude polyporin in Calcutta hospitals (1944 to 1946) were sufficiently encouraging to justify trials on a larger variety of cases preliminary to comparing polyporin with other bactericidal agents.

Atkinson (158) has tested the antibacterial activity of 200 kinds of mushrooms and toadstools collected in South Australia. The water extracts of several species of *Cortinarius* and one of *Psalliota* showed activity. *Cortinarius rotundisporus* and *Psalliota xanthoderma* were found to inhibit the growth of *Staphylococcus aureus*, *Eberthella typhosa* and *Mycobacterium phlei*. In a survey of 230 species of basidiomycetes, Mathieson (159) partially purified an agent from *Psalliota* sp. which is active *in vitro* against *Staphylococcus aureus*, hemolytic streptococci, *Eberthella typhosa* and *Shigella* (Flexner III). The compound is not toxic for mice. Further evidence for the activity of *Psalliota xanthoderma* is supplied by

Wilkins (160) who tested many basidiomycetes collected in Great Britain. Two strains of *P. xanthoderma* inhibited *S. aureus* and *E. coli*. Inhibition of both of these test organisms occurred with only 14 of 787 basidiomycetes and larger ascomycetes tested.

Hollande (161) has reported an active agent from an edible fungus identified as *Clitocybe candida* or *C. gigantea*. Subsequent studies on this substance, which he named CLITOCYBIN (162), have shown it to be remarkably active against *Mycobacterium tuberculosis*, *Brucella abortus*, *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*. Partially purified extracts of clitocybin were innocuous to guinea pigs. Most remarkable was the observation of progressive and total lysis of *M. tuberculosis* in infected guinea pigs treated with the antibiotic.

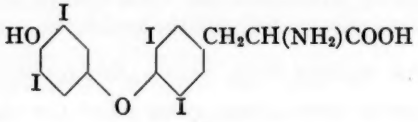
Antibiotics From Algae and Lichens

Crude extracts from culture solutions of the algae *Chlorella vulgaris* and *Chlorella pyrenoidosa*, were reported by Pratt *et al.* (163) to be bactericidal for *Staphylococcus aureus* and *Escherichia coli*, and active against *Streptococcus pyogenes*, *Bacillus subtilis*, and *Pseudomonas aeruginosa*.

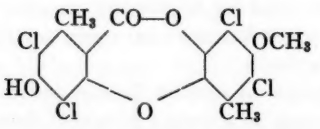
Antibiotic studies on various lichens indicate that this group may prove promising as a source of useful therapeutic agents. Of approximately one hundred species of lichens tested for antibiotic activity against various bacteria by Burkholder & Alexander (164), fifty-two were found to inhibit *Bacillus subtilis* or *Staphylococcus aureus* or both. Gram-negative bacteria in general were found to resist the agents found active against gram-positive types. Barry & McNally (165) have investigated the effect of various lichen acids and derivatives on the growth of tubercle bacilli. They found that the half-esters and half-amides from ROCELLIC ACID (α-methyl-α'-n-dodecyl succinic acid) isolated from the lichen, *Lecanora sordida*, completely inhibited the growth of tubercle bacilli at a dilution of 1:500,000.

Barry (166) noted that the chemical constitution of thyroxine (XVI) closely resembles that of DIPLOICIN, (XVII) isolated by Nolan in 1935 from the lichen *Buella canescens*. Since diploicin is insoluble in water, water solubility was obtained by hydrolysis of the ester linkage to open the depside ring. A neutral solution of this compound was found to inhibit *Mycobacterium smegmatis* at 1:70,000 and *M. tuberculosis* and *Corynebacterium diphtheriae* at

1:100,000. Thyroxine, per se, exerts no effect on the growth of *M. tuberculosis in vitro*. However, it is known that tuberculosis rarely develops in association with hyperthyroidism, that thyroidectomy increases susceptibility to the disease, and that the thyroid gland is seldom attacked. In a review of antitubercular compounds Barry (167) describes new derivatives of diploicin which he has tested for tuberculostatic activity. Slight alterations of the diploicin molecule have produced some derivatives which are highly active and others which have no activity. A free phenolic hydroxyl group in each ring appears to be necessary, and the balance is clearly a delicate one.



XVI



XVII

One of the most promising studies on antibacterial substances from lichens is that of Marshak (168). From the lichen *Ramalina reticulata*, an epiphyte growing along the west coast of North America, he has isolated a crystalline acid in about 0.15 per cent yield. It analysed as $C_{16}H_{14}O_6$ and has one carboxyl and no methoxy groups. It is active against certain gram-positive bacteria and a wide variety of acid-fast organisms. The drug in oil suspension may be injected subcutaneously into 350 to 400 gm. guinea pigs at daily rates of 10 to 20 mg. for three weeks without evident toxic reactions. When the drug is so administered to guinea pigs infected intraperitoneally with human tubercle bacilli, the progress of the disease is definitely retarded. Marshak's promising results and the studies of Barry and others indicate that the many lichen acids already isolated and characterized should be thoroughly reexamined for possible antibiotic activity, especially with reference to their ability to inhibit the growth of acid-fast organisms.

Antibiotics From Higher Plants

The search for antibiotics in higher plants has not met with the dramatic success that has attended the investigation of microorganisms. Only a few of the plant antibiotics thus far reported have been crystallized. In a survey of plant substances inhibitory for malarial parasites, bacteria, and viruses, Carlson *et al.* (169) tested extracts from more than 200 wild plants collected in Oregon. Saline extracts of buttercup, sagebrush, dwarf water leaf, mountain pasque, and juniper were active against *Staphylococcus aureus*. Mountain pasque and buttercup gave off vapors and volatile oils which were bacteriostatic or bactericidal for a variety of gram-negative and gram-positive bacteria, and an extract of mountain pasque was found to protect mice heavily infected with pneumococci.

In testing extracts of 1,100 Australian plants, Atkinson (170) found that fifty inhibited *Staphylococcus aureus* but that only four of the fifty (*Drosera Whittakeri* and three species of *Persoonia*) also affected *Eberthella typhosa*. Considerable purification of the substance from the berries of *Persoonia* has been made.

The antibacterial effect of onions and garlic has been attributed to a variety of substances. The name allicin, formerly applied to an antibacterial substance obtained from *Allium sativum* (garlic) by Cavallito *et al.* (171) was withdrawn in view of possible confusion with established medicinal products. These workers found that whole garlic contains the active principle in the form of a thermostable precursor which is rapidly broken down in the presence of an enzyme and water to yield the active agent (XVIII) when the garlic cells are crushed. The reaction is believed to proceed as follows:

$$\text{Precursor} + \text{enzyme} \xrightarrow{H_2O} C_3H_5SOSC_3H_5$$

XVIII

The active agent is a colorless oil irritating to the skin and has a very strong garlic odor. Toxicity for mice ranges between 60 to 120 mg. per kg. depending on the mode of injection. Özek (172) found that volatile compounds of garlic destroyed intestinal bacteria *in vitro*, but whole garlic had no effect on the bacterial flora of human intestines *in vivo*, when ingested daily for fifteen days.

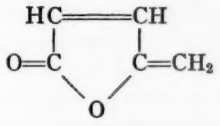
Rao *et al.* (173) found that garlic extracts prepared by a slight modification of the method of Cavallito *et al.* (171) effected complete inhibition of growth of human tubercle bacilli.

Hooker *et al.* (174) have isolated from the roots of several crucifers (turnip, black and white mustard and horseradish) two compounds, β-phenethyl-isocyanate and allylisothiocyanate. These compounds markedly inhibit the spore germination of *Plasmodiophora brassicae*, the organism responsible for the disease known as clubroot in these plants. Previous investigators of plant antibiotics have indicated that resistance of some plant varieties to phytopathogens might be related to the presence of specific substances in the juices of these plants. To test the validity of these postulates, Little & Grubaugh (175) extracted juices from disease resistant varieties of bean, corn, cabbage, wild mustard, cucumber, tomato, and cauliflower. Seitz-filtered extracts were tested against *Staphylococcus aureus*, *Eberthella typhosa*, *Salmonella paratyphosus* A, *Escherichia coli*, and certain specific plant pathogens against which the plants are resistant. The juices were found to be more active against human pathogens than against plant pathogens. Only corn, cucumbers, wild mustard, and cabbage demonstrated any activity against the bacterial phytopathogens and only corn and tomato plant juices inhibited the growth of plant pathogenic Fusaria.

Seegal & Holden (176) have investigated the antibacterial principle from buttercups extensively. The growth of various gram-negative and gram-positive pathogens, *Mycobacterium tuberculosis*, and certain pathogenic yeasts was inhibited by the juice, which was found to be quite toxic for experimental animals. The active principle from buttercups is believed to be identical with PROTOANEMONIN, $C_5H_4O_2$, which Baer *et al.* (177) have extracted from the plant *Anemone pulsatilla*. This compound, (XIX) a pale yellow oil which rapidly polymerizes to anemonin and higher polymers, was first synthesized by Japanese chemists in 1922.

Crystalline antibiotics have been isolated from two members of the Compositae family. One of these is CREPIN, $C_{14}H_{16}O_4$, isolated and tentatively identified by Heatley (178), from flower petals of *Crepis taraxacifolia* (Thuill). It is probably a β-γ-unsaturated lactone, most active against gram-positive bacteria, but it is too toxic for *in vivo* experiments in animals. The second active

agent, found in *Arctium minus* (common burdock), was purified by Cavallito *et al.* (179) who thought it to be an unsaturated lactone, $C_{15}H_{20}O_5$. The crystalline compound, weakly active against gram-positive bacteria and ineffective against gram-negative types, is toxic for mice when injected intravenously in doses of 90 mg. per kg. Subcutaneous administration of 50 to 250 mg. per kg. in mice infected with *Streptococcus hemolyticus* C-203 did not protect the animals. Abraham *et al.* (180) have crystallized a substance from *Arctium minus* and *Onopordon tauricum* which closely resembled the compound purified by Cavallito *et al.* (179). However, x-ray diffraction patterns produced by the two compounds differed sharply, and Abraham and co-workers have suggested that their compound may be isomeric with that of Cavallito and co-workers.



XIX

From *Asarumcanadense* var. *reflexum* (wild ginger) Cavallito & Bailey (181) have isolated two purified products: Compound A which analyzed as $C_{21}H_{20}O_8N_2S$ and Compound B which appears to be $C_{16}H_{11}O_7N$. Both are active against gram-positive bacteria. Compound A, which is much more active than B, inhibits *Staphylococcus aureus* at 1:500,000 dilution. Doses of 5 mg. per kg. of A, given intraperitoneally in mice, are lethal after two to three days.

Abraham *et al.* (182) have isolated a crystalline compound from *Spiraea aruncus* L. (goat's beard) which analyzed as $C_{10}H_{14}O_4$. Its properties suggest that an α, β-unsaturated lactone ring may be present in the molecule. The antibacterial action is very weak, and *Escherichia coli* and *Staphylococcus aureus* are not inhibited beyond a dilution of 1:4,000.

An active bactericidal agent present in *Larrea divaricata* Cav. has been characterized by Waller & Gisvold (183) as NORDIHYDROGUAIARETIC ACID. It is active, *in vitro*, against a number of gram-positive and gram-negative pathogens. In the form of freshly prepared tinctures (acetone, alcohol and water) it exhibits marked skin sterilizing properties.

Goth (184) has reported that DICOUMAROL, 3,3′-methylenebis (4-hydroxycoumarin) has marked antibacterial action against various gram-positive bacteria. Compounds like 1,4-naphthoquinone and others which possess vitamin K activity, and which are known to counteract the hemorrhagic effects of dicoumarol, failed to antagonize the growth inhibition of *Staphylococcus aureus* in the presence of the latter. In possessing both hemorrhagic and antibiotic properties this substance is indeed unique.

From the brown portions of the plant *Tillandsia usneoides* (Spanish moss), Weld (185) obtained a crude extract effective against all gram-positive bacteria tested, and against *Cryptococcus hominis*, but it had no effect on gram-negative organisms.

One of the most interesting plant antibiotics is TOMATIN, formerly called lycopersicin by Irving *et al.* (186, 187). Although the best source of the active agent appears to be the Red Currant tomato plant, it is found to some extent in plants of all commercial tomato varieties. This substance, which has not yet been purified, inhibits the growth of various gram-positive and gram-negative bacteria and is highly fungistatic against certain molds and pathogenic yeasts. Fontaine *et al.* (188) in preliminary toxicity tests on crude tomatin, found that intravenous, intraperitoneal or subcutaneous injections of 1 mg. in guinea pigs induced no unfavorable reaction. However, 10 mg. administered by either of the latter two methods cited above were not fatal, but did induce unfavorable reactions.

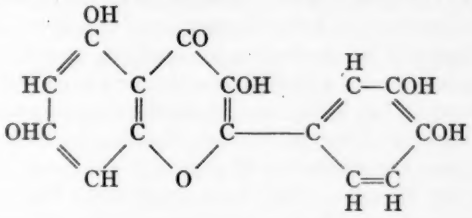
In a survey of antibiotics from higher plants in China, Chen *et al.* (189) found that extracts of the water chestnut, *Eleocharis tuberosa* contained an agent which inhibited various gram-positive bacteria. The active substance has been named "PUCHIIN" from the Chinese characters for *E. tuberosa*.

Southam (190) has shown that an aqueous extract of the heart wood of western red cedar (*Thuja plicata* D. Don) is bacteriostatic for a wide variety of gram-positive and gram-negative bacteria and various fungi. The agent is thermostable, resists treatment with strong acid or alkali but is inactivated by blood, serum, or cysteine. Large doses of the extract did not injure mice or rabbits. No *in vivo* activity against infections has yet been demonstrated.

Rao *et al.* (191) reported that alcoholic extracts of different parts of the plant *Moringa pterigosperma* Gaertn. showed pronounced antibiotic activity against a variety of gram-positive and

gram-negative pathogens and *Mycobacterium tuberculosis*. The active principle named PTERIGOSPERMIN is an alcohol soluble, water insoluble oil with a highly irritating smell.

QUERCETIN (XX), also known as meletin, flavin, sophoretin and tetra-hydroxyflavonol, occurs naturally in a variety of vegetable materials including the rinds of many fruits. Naghski *et al.* (192) have found that quercetin has antibacterial properties at



XX

pH 5.2 to 7.0 but is inactive above pH 7.0. Of the organisms tested, those most affected were *Staphylococcus aureus*, *S. albus*, *Aerobacillus polymyxa* and *Brucella abortus*. Activity was lost in the presence of serum and iron, but not in the presence of cysteine.

Mechanism of Action of Antibiotics

Within the past two years, considerable effort has been made to determine the mechanisms involved in the inhibition of microbial growth by various antibiotics. Information of this sort is lacking for many of the recently described antimicrobial substances. In other cases nothing has been reported beyond the fact that an agent is bacteriostatic, bactericidal, or bacteriolytic; or that it is fungistatic, or fungicidal. Many investigators have approached the problem by *in vitro* chemical inactivation studies involving the use of various oxidizing and reducing agents.

Hotchkiss (193) found that gramicidin may act on bacteria by inhibiting polysaccharide accumulation, phosphate uptake and cell multiplication. Lysis by tyrocidine is a secondary phenomenon, following slowly after a rapid primary injury which is apparently some alteration of the cellular membrane, or surface. Blinnikova (194) reported that tyrothricin inhibited not only enzymic dehydrogenation of glucose, but also that of ethyl alcohol and glutamic, lactic, fumaric, and acetic acids. The dehydrogena-

ses and the growth of a particular microbe were inhibited by exactly the same concentration of tyrothricin.

Various workers have found that streptomycin is inactivated *in vitro* by a number of reducing agents and other chemicals (195, 196). Cysteine appears to be among the most active of the reducing agents in this respect. This suggests that streptomycin may exert its antibacterial effect by interfering with the normal functions of sulfhydryl groups in bacterial metabolism. Van Dolah & Christenson (197) feel that *in vitro* inactivation of streptomycin may not logically be ascribed to an oxidizing or reducing mechanism but results from a specific reaction or an interference mechanism. Henry *et al.* (198) investigated streptomycin inhibition of a large number of enzyme systems of microorganisms and found only one (aerobic oxidation of glycerol) that was affected by streptomycin. However, they have emphasized that this work is preliminary and possibly the knowledge sought cannot be learned from nondividing bacterial cells, a fundamental fact too frequently overlooked. Benham (199) studied the effect of streptomycin on the oxygen uptake of *Eberthella typhosa*, and found that it was greatly increased. Thus it may interfere with certain cell functions so that normal synthesis of cell components does not take place. Hobby & Lenert (200) have observed that streptomycin is primarily bacteriostatic and is effective against slowly dividing bacterial cells only if high concentrations of the drug are present. Low concentrations of the drug will inhibit bacteria which normally multiply rapidly in its absence, but the same concentrations are ineffective against bacteria which are either stationary or dividing only slowly.

Abraham (201) studied the effect of proactinomycin on *Staphylococcus aureus* and found that low concentrations of the drug lengthened the lag phase of *S. aureus*, and that filtrates from young cultures counteracted this effect. Thus, proactinomycin may interfere with the synthesis or use of some diffusible substance produced by the cells before rapid growth can occur. The same investigator (149) found that *nor*-mycophenolic acid inhibits *S. aureus* in a manner similar to that of proactinomycin.

Gale & Taylor (202) have listed four effects of penicillin on *Staphylococcus aureus*, namely: assimilation of glutamic acid is prevented, the respiration progressively fails, the cells become nonviable, and lysis occurs after several hours. They found that

prevention of assimilation of glutamic acid precedes both respiratory failure and lysis of cells. Since glutamic acid assimilation is prevented by penicillin, it is postulated that the drug combines with or produces a reorganization of the cell walls which blocks the assimilatory mechanism. Using sodium ribonucleinate as a substrate, Krampitz & Werkman (203) observed that oxygen uptake of lyophilized cells of *Staphylococcus aureus* and other bacteria is completely inhibited in the presence of penicillin. Similarity of the oxygen uptake curves representing the sodium ribonucleinate and endogenous activity suggests that penicillin inhibits dissimilation of cellular ribonucleic acid. Oxygen consumption in the presence of a variety of other substrates was not affected by the antibiotic.

Michaelis & Thatcher (204) found that oxygen uptake by *Staphylococcus aureus* with either lactate or glucose as the substrate is depressed almost immediately upon the addition of citrinin, either in the presence of living or dead cells. The degree of inhibition is proportional to the concentration of citrinin present.

The mechanism whereby claviformin and penicillic acid inactivate bacteria is thought by Geiger & Conn (205) to result from their reaction with the sulfhydryl groups of bacterial enzyme systems, or with sulfhydryl-containing metabolites essential to the bacteria. Since the above compounds are unsaturated lactones, other antibiotics in this chemical group including protoanemonin, crepin, $C_{15}H_{20}O_5$ from *Arctium minus* and $C_{10}H_{14}O_4$ from *Spiraea aruncus* L. may exert antibiotic action by the same mechanism.

Goth (85) has advanced the hypothesis that aspergillic acid inhibits bacterial growth by the formation of an aspergillic acid-ferric iron complex which makes ferric iron unavailable to the organism. This theory is supported by the fact that ferric ions interfere with the antibiotic activity of the agent and that this reaction precipitates ferric ions in solution. Potentiation of the activity of aspergillic acid by bismuth (86) is thought to be due to the simultaneous interference with sulfhydryl groups by the bismuth and the binding of ferric ions by aspergillic acid.

There are several *p*-quinones among the naturally occurring antibiotics, namely: fumigatin, spinulosin, citrinin and actinomycin. Hydroquinones are represented by puberulic acid and gentisyl alcohol, and javanicin is a substituted naphthoquinone. Geiger (206) has extended his study on the mode of action of α, β-unsaturated ketones (represented by claviformin and penicillic

acid) to include certain synthetic *p*-quinones, which contain two α,β-unsaturated ketone groupings in each molecule. Various synthetic hydroquinones with antibacterial action were included also because of their close chemical relationship to quinones. His data show that the weak bacteriostatic action which various synthetic benzoquinones and naphthoquinones exert against gram-negative organisms was antagonized by cysteine and thioglycollate, whereas their marked action against gram-positive organisms is not prevented by these agents. He was able to show that quinones are still active against gram-positive organisms after all the hydrogen atoms are replaced by substituting groups, hence sulfhydryl compounds cannot react with them. This further indicates that the antibacterial action of quinones against gram-positive cells does not result from an interaction with sulfhydryl systems in the bacterial cell.

With the evidence obtained thus far on the modes of antibacterial action, it appears to us that highly reactive antibiotics interact with a number of enzyme systems or metabolites essential to the bacterial cell. We heartily agree with Wintersteiner (207) that the known complexity of bacterial metabolism and enzyme systems on the one hand, and the great diversity of chemical structure encountered among antibiotics on the other hand greatly reduces the possibility that their antibacterial action is due to a single mechanism, as envisaged in the sulfhydryl hypothesis.

Various organisms are known to become antibiotic-fast, such that their progeny resist many times the antibiotic concentration required to inhibit completely the parent cells. The development of resistant strains often occurs in patients where subminimal amounts of antibiotic are administered on initial treatment. This problem is less likely to occur in current streptomycin or penicillin therapy since adequate supplies of these drugs are generally available for the treatment of many susceptible pathogens.

Sullivan *et al.* (208) developed streptomycin-, streptothricin-, and penicillin-resistant strains of *Staphylococcus aureus* and *Escherichia coli*. They found that the strain of *E. coli*, which was made resistant to streptothricin was also more resistant to streptomycin than the parent culture; however, the reverse was not true. With the exception noted, the development of resistance to one antibiotic agent did not result in increased resistance to either of the others. Klimek *et al.* (209) found that resistance in a strain

of *Staphylococcus aureus* could be rapidly developed against penicillin, streptomycin, pyocyanin and the active principle of *Asarum canadense* var. *reflexum*. Only slight resistance could be developed against gliotoxin, aspergillic acid, and the active principle of *Allium sativum*. Increase in resistance to any one of the antibiotics in no way affected its sensitivity to any of the others. Reversal to a sensitive state was found to be rapid, regardless of time required to develop high or low degrees of resistance. Development of resistant strains as a means of differentiating antibacterial substances has been suggested by Eisman *et al.* (210). However, there are limitations in this method as shown by the fact that three different penicillins cannot be differentiated by a penicillin G resistant strain of *Staphylococcus aureus*.

Waksman (68) has appropriately suggested that antibiotic-fast organisms might be eliminated in clinical practice if a mixture of antibacterial agents is simultaneously injected. It is believed improbable that an organism could rapidly and simultaneously build up resistance to two or more agents, hence lower concentrations of each would be required than if a single substance were used.

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CHEMOTHERAPEUTIC AGENTS

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Any attempt to deal adequately in a few pages with all the striking and manifold current advances in chemotherapy would be futile. Progress has been too energetic in too many directions, and this review will therefore be no more than an appraisal of the scope of the subject, some of the broader recent trends of its progress, and its general relationship to human affairs. This will leave various component and contributory aspects of chemotherapy for more detailed consideration elsewhere in this and in succeeding volumes of these Reviews. Such being the aim, references to published work will be severely limited in number, though some attempt will be made to guide the reader to other and more comprehensive surveys and bibliographies of special aspects of chemotherapy.

The Scope of Chemotherapy

There is no general agreement as to an exact definition of chemotherapy. Some would include, for example, the use of synthetic or nonsynthetic substances for endocrine disorders (1) or other constitutional, noninfective, disturbances (2), or the administration of antidotes to cases of poisoning (3). Most workers, however, confine the term to the treatment of infections and of cancer (including blood dyscrasias bearing some of the features of cancer) by chemical compounds. However, even this still leaves the boundaries of the subject ill-defined, since any substance administered for therapeutic purposes must, after all, bear some chemical constitution. Are we to include serum therapy, for example, in chemotherapy? Difficulties still remain if we attempt to define the subject more closely by referring only to chemical substances of relatively simple composition, whose structure is exactly known. Did penicillin therapy become a part of chemotherapy only at, and not before, the particular moment when the chemical structure of penicillin was determined? Are we to exclude vaccine therapy, whilst admitting the administration of synthetic antigens of known composition, such as those of Goebel (4), which give rise to antibodies protective against pneumococcal infection? It is true that these

particular antigens consist only in part of substances of exactly known chemical composition, since the latter must be combined outside the body with globulins (whose precise chemical composition is of course not known) in order to constitute the complete antigen. In the work of Gell, Harington & Rivers (5) the only substances injected were chemical compounds of quite simple, known, composition. Antibodies, however, appeared in the circulating blood, in response doubtless to true antigens presumably formed by some manner of combination between the chemical substances and serum proteins of the animal. It is easy to visualise development of such studies to the point where animals may be protected from infection by similar means. The fact that treatment by such procedures would be prophylactic rather than therapeutic does not affect the issue, since preventive and curative aims are equally legitimately a part of chemotherapy. Neither is the argument affected by the fact that, in the case of these agents, immune processes would provide the overriding and determining contribution towards the final effect. Immune processes play a fundamental role in innumerable cases which no one would hesitate to class as true instances of chemotherapeutic action. No doubt, however, the answer here is that if the antibacterial effect is dependent upon an immune response directed, by means of specific antibodies, against the therapeutic (or prophylactic) agent itself, then we must exclude the example from the scope of chemotherapy. However, there is in fact a form of chemotherapeutic research, of a less strictly immunological character, in which drugs are designed not for the purpose of themselves destroying the parasite, but of helping the host to do so. Thus, Robinson (6) has outlined the steps which have led to a strategy which abandons a frontal attack on the tubercle bacillus, in favor of an attempt to alleviate the pathological consequences of infection, in the hope of thereby encouraging the host's immunity processes.

Difficulties of exact definition also arise from the fact that types of infection and parasitism range by stages from those of deep seated location in the blood or internal organs, at one extreme, to infestation by ectoparasites, or even to the visitations of blood sucking diptera, at the other. At which point in the following sequence of examples does the term chemotherapy cease to be applicable: treatment for malaria; for amoebic dysentery; for intestinal helminthiasis; for scabies; for louse infestation (as by DD

T); and for mosquito visitations (as by insect repellents)? And if the application of insecticides or insect repellents to the skin be rejected as instances of chemotherapy, then is this also to be the verdict in the case of successful attack upon blood sucking arthropods by oral treatment with chemical substances, so that concentrations noxious to the arthropods are attained in the host's circulating blood, as demonstrated by Lindquist *et al.* (7) and De Meillon (8)? It would be wisest then, in accepting any definition of chemotherapy, to accept also the qualification that the range of its applicability cannot be precisely defined.

There will necessarily be very considerable gaps in this Review. Some of the aspects to which only the scantiest (or no) reference will be made are the chemotherapy of tuberculosis and leprosy, virus and rickettsial diseases, amoebic dysentery, trypanosomiasis, leishmaniasis, helminthiasis, and cancer. There will be practically no attempt to deal with antibiotics. The most serious gap in the discussion of theoretical aspects will be omission of any reference to acquired drug resistance, a subject which has thrown much indirect light on modes of drug action, and which is of sufficient complexity and importance to deserve treatment in a separate contribution. It has recently been briefly reviewed by Selbie (9).

Recent Trends

It is remarkable that progress of the very highest order, in elucidating the fundamental bases of chemotherapy, should have been achieved during the actual progress of the recent world war. But it was, of course, not merely by the impetus of war alone that chemotherapy has pressed so far forward in recent years. The achievements of this period would not have been possible had the stage not been well set in the years immediately preceding the war. The introduction of sulfonamide therapy for bacterial infections, by Domagk (10) in 1935, at once opened a rich field for experiment and achievement, which many had previously believed to be almost the exclusive preserve of those primarily interested in diseases caused by protozoa and spirochaetes. The development of sulfonamide therapy practically coincided, independently, with a quickening of interest in the metabolism of bacteria, fostered in particular, or at least initially, by the (British) Medical Research Council's Unit for Bacterial Chemistry, under Fildes (11). The convergence of studies on bacterial metabolism with those on chemotherapy

was consummated by the classical interpretation by Woods (12) of the mode of action of sulfanilamide, on the basis that it blocks the utilisation, by the bacterial cell, of *p*-aminobenzoic acid, which was believed, and later proved by Rubbo & Gillespie (13) and others, to be essential for the nutrition of many bacterial species. This theory of the mode of sulfonamide action has been generally accepted, although Sevag and co-workers (14, 15) have proposed as an alternative hypothesis that sulfonamide action is dependent on an inhibition of respiratory enzymes, this inhibition being subject to reversal by *p*-aminobenzoic acid.

Chemotherapy by blocking specific nutrients.—Woods' publication appeared almost at the same time as a lucid exposition by Fildes (16) of the newer orientation of chemotherapy, in the light of current knowledge of bacterial nutrition. In this "rational approach to chemotherapy" to use Fildes' own words, chemotherapeutic activity was conceived in terms of interference at some particular stage in the progression of metabolic reactions involved in the parasite's nutrition. This interference takes the form of inactivation or displacement of a metabolite essential to the parasite's nutritional economy

> "(*a*) by oxidizing a substance which requires to be reduced; (*b*) by molecular combination, forming an inactive product; or (*c*) by competition for an enzyme associated with the esssential metabolite."

The last-named mechanism, in which inhibitor and metabolite would characteristically be structurally similar, was clearly not put forward as the only, or necessarily the most effective, means by which a specific metabolite might be attacked. Nevertheless, its clear enunciation, and the support it received from the studies on sulfanilamide action, immediately stimulated attempts to discover chemotherapeutic activity among compounds structurally related to other essential metabolites of known composition. This development of chemotherapeutic research was, in reality, merely an application to chemotherapy of a principle which had been well-established in biological chemistry since the turn of the century—the principle of interference with enzyme-action by the interposition of a substance structurally related to the substrate, coenzyme, or product of the action concerned. Such an approach to the design of new chemotherpaeutic agents is especially attractive, as a radical departure from the empirical methods of the past, which had

involved the trial of endless variants of a chemical type previously found by chance to possess some therapeutic powers. It is true that by the latter, traditional, means some correlation between chemical structure and activity may be expected to become apparent, in the course of time, and may then serve, to some extent, as a guide for the selection of further compounds for trial. These correlations are, however, established *a posteriori*, generally without the concomitant emergence of any sound rationale linking chemical structure and therapeutic activity, whereas in the newer approach, the correlations, of a very intimate and rational nature, are the starting-points in the selection of new compounds, and are, in fact, the reasons for their selection.

We may expand Emil Fischer's famous enzyme-substrate lock and key analogy by regarding the nutritional path along which the parasite must travel as beset with a number of locked gates, for each of which the right key is required. For chemotherapy by competitive inhibition of enzymes, the pattern of any one of these keys must be close enough to the right one for the lock to be engaged, but sufficiently dissimilar for the lock to be jammed, an analogy used in a recent review by Welch (17). That there are so many locked gates for the cell to negotiate in its nutritional progress (and that the features of so few of the locks are yet known) is a measure of the opportunities open to chemotherapy by this approach. One of the serious difficulties is, however, that metabolites essential for the parasite are likely to be essential also for the host, indeed, as van Niel (18) has pointed out, the identification of growth factors for bacteria is coming to be almost a regular starting point for the discovery of substances essential for higher organisms. This implies the danger that substances lethal to the parasite may, by similar if not identical mechanisms, be toxic also to the host. On the other hand, the essential metabolite may be present as a normal component of the body fluids or tissues in such excess that its neutralisation or displacement to the extent of injury to the host may be very unlikely. In this event, however, it may not be posible to produce a sufficient *in vivo* concentration of the inhibitor (i.e., the potential chemotherapeutic agent) to enable it to compete effectively with the essential metabolite for fixation on the parasite-cell, and the attempt at producing a chemotherapeutic effect is thus foiled. McIlwain (19) examined sulfanilamide from this point of view, and showed that its activity *in vitro* and *in vivo*

are entirely comparable, in that both circumstances demand the same ratio of sulfanilamide to *p*-aminobenzoate to produce inhibition of bacterial growth. This aspect of chemotherapy was advanced in other directions with considerable skill by McIlwain, and he (and many other workers concurrently and since) have demonstrated inhibition of bacterial growth by a number of compounds selected merely on the grounds of structural similarity with known essential metabolites. Of these growth inhibitors, pantoyltaurine, structurally related to the essential metabolite pantothenic acid was a notable example, as had already been shown independently by Snell (20, 21) and by Kuhn *et al.* (22). McIlwain (23) postulated pantoyltaurine would possess therapeutic activity against hemolytic streptococcal infections on ascertaining that the ratio pantoyltaurine: pantothenate required for bacteriostasis *in vitro* could probably be attained *in vivo*. The prediction of chemotherapeutic activity was brilliantly successful, in tests by McIlwain & Hawking (24), and the rationale of its mechanism was supported by showing that the therapeutic effect in rats could be annulled by the concomitant administration of pantothenate to the pantoyltaurine treated animals. Also, the therapeutic effect could not be produced in mice, whose pantothenate blood level is normally much higher than that of rats. The activity in rats was not of a high order, but the special importance of the work is that it was a practical demonstration of an entirely new method for the discovery of chemotherapeutic agents. It naturally led to the search for chemotherapeutic activity by other modifications of pantothenic acid, and a high degree of such activity in mice has now been claimed for substituted pantoyltauramides by White *et al.* (25).

It is, of course, obvious that progress along such lines is radically dependent upon an accurate knowledge of the complex nutritional requirements of the particular parasite (and host) concerned. Such knowledge in regard to bacteria has only begun to accumulate to any significant extent within about the past decade, and cannot therefore yet be regarded as in more than its (lusty) infancy; similar knowledge in regard to parasitic protozoa is in an even more rudimentary stage, and indeed investigations upon the nutritional requirements of this class of parasite are among the researches ancillary to chemotherapy which are most urgently called for at the present time. Recent knowledge of this kind in regard to free-living protozoa has been reviewed by van Niel (18), Snell (26), and

by Lwoff (27) in the present volume.

There are various special obstacles in the way of elucidating the nutritional requirements of parasitic protozoa. There is, for example, the fact that many of the more important of these parasites are considerably more difficult to cultivate in the test tube than are strains of bacteria, and some have in fact not yet been maintained in this way. Then there is the fact that the culture forms of some protozoa differ considerably from the forms which thrive in the vertebrate host. For example, the nutritional requirements of the crithidia of *Trypanosoma cruzi* cultures, or of the leptomonads of *Leishmania* cultures, must surely differ considerably from those of the trypanosome or of the leishman donovan stages, respectively, in the vertebrate host. The culture forms are akin to the forms which occur within the insect vector, and quite apart from the indirect evidence provided by obvious morphological and by known physiological differences, it is inherently rather unlikely that the nutritional demands of a parasite would be the same in the intestinal tract of say a reduviid bug or of a sandfly as they are in the body fluids or cells of a warm-blooded vertebrate. In the case of *Entamoeba histolytica* an obstacle of quite a different kind in the way of determining the parasite's essential nutrients, is that cultures cannot easily be maintained in the absence of bacteria.

In the case of successful *in vitro* culture of malaria parasites in red blood cells (28, 29, 30) or in tissue cells (31), the complex nutrients provided by these cells cannot at present be accurately analysed. Nevertheless the procedure of deliberately searching for variants of known essential growth factors has already been applied with some success in the search for new antimalarials.

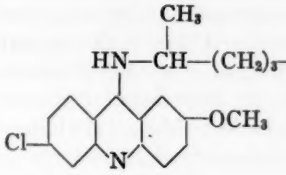
Antimalarials.—Early in the war it was fully appreciated, on both sides of the Atlantic, that one of the factors upon which victory might depend would be our ability to control malaria among the huge armies to be deployed in endemic areas. The prospects of such control were suddenly thrown into serious jeopardy in 1942 by the loss of the Dutch East Indies, the source of about 95 per cent of the world's cinchona. Fortunately, however, the folly of depending solely on quinine for the chemotherapeutic mass control of malaria had already been well understood at the very outbreak of war, and great energies were accordingly thrown into the manufacture and investigation of quinacrine (known as mepacrine in

Britain; formerly the German atabrin). Nevertheless, the risk was great, for there was little precise knowledge of the best way of using quinacrine, or of its exact therapeutic or toxic potentialities. In an army group undergoing suppressive antimalarial treatment an outbreak of serious toxic reactions at a critical moment might have spelt disaster to a whole campaign, and there were, in fact, a number of disquieting though equivocal reports, from time to time, of local incidence of toxicity tentatively attributed to quinacrine, in various theatres of the war, notably in North Africa in 1943 (32). Shannon *et al.* (33) established correlations between activity and blood levels of quinacrine, as a basis for its administration on rational lines, and further work both by American and British investigators finally established not only that it is essentially a nontoxic drug when properly used, even over long periods of suppressive medication under combat conditions, but that it would in fact be the drug of choice even if quinine were freely available. It is as effective as quinine (though neither compound is radically curative) in vivax malaria, whilst it is more reliable than quinine as a prophylactic and as a radical curative agent in falciparum malaria (34, 35). Outstanding among the researches leading to these conclusions were those conducted in Australia by Fairley (36).

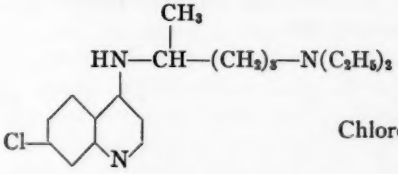
However, it was obviously not safe merely to gamble upon a successful outcome of the investigations with quinacrine, and intensive efforts were therefore concurrently put in train to discover new antimalarials. In the United States the search proceeded in the main, though far from exclusively, along the more traditional lines of chemical development in relation to chemotherapy. This refers only to the technical approaches, for there was indeed nothing traditional about the impressive organisation and scale of the work—truly one of the monumental examples of widely ranging and willing cooperation achieved under the stimulus of war. This is fully described, together with detailed results, in a monograph edited by Wiselogle (37). Over fourteen thousand compounds were screened for antimalarial activity, and the chemical types which retained the lead as the most promising were derivatives of quinoline, this nucleus having already provided the pioneer of successful synthetic antimalarials, in pamaquine (formerly the German plasmochin).

The quinoline categories which have yielded the most promis-

ing compounds are the 4-amino- and the 8-aminoquinolines. The outstanding member of the former group is chloroquine (SN 7618); this may be regarded as an abbreviation of quinacrine, which it



Quinacrine



Chloroquine

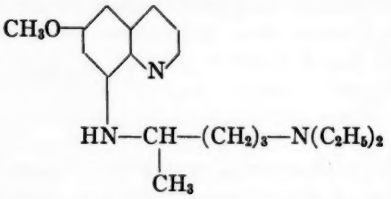
closely resembles in chemotherapeutic properties, though it has the additional advantage of effectiveness in dosages administered at widely spaced intervals, or in very short treatment courses (38, 39).

The special virtue, which, it is hoped, may lie latent in the 8-aminoquinolines is the much sought, and much required, ability to cure benign tertian malaria, a feature not consistently exhibited by any drug yet tested. The basis of this expectation lies in the fact that pamaquine is an 8-aminoquinoline and that, although it is not curative in a high proportion of cases of benign tertian malaria when given alone, it now seems clear, in confirmation of earlier claims by Sinton *et al.* (40, 41), that the cure rate may be very high when it is given in conjunction with quinine (42, 43, 44). The member of this group selected as the most promising was pentaquine (SN 13, 276). Its performance against vivax malaria is similar to that of pamaquine in that it cures a high proportion of cases when administered together with quinine, though it also does not reach the ideal of consistent curative properties (for this type of malaria) when given alone (45).

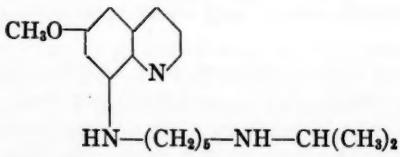
Among other quinoline types in which a fair degree of activity has been found are two categories of 4-quinoline methanols, one

modelled on the piperidyl prototype of Ainley & King (46), and the other on the dialkylaminomethyl prototype of King & Work (47).

The naphthoquinones (which share with other quinones a conspicuous degree of antibacterial activity) have also provided a number of antimalarial substances (48, 49) of somewhat less promise, and another quite unrelated type is the metanilamide group, explored in particular by English *et al.* (50), Brackett *et al.* (51) and Hughes & Brackett (52), the most hopeful member being metachloridine, i.e., 2-metanilamido-5-chloropyrimidine. In contrast to the sulfonamides, which have very slight activity against *P. cathemerium* in the canary, the metanilamides are highly active against these infections (52), but their action on human malaria has so far proved disappointing (49).



Pamaquine



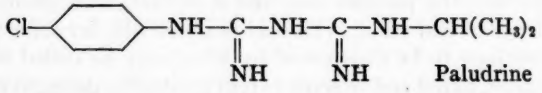
Pentaquine

That antimalarial agents would eventually be discovered by deliberately modelling compounds to the pattern of essential growth factors was foreshadowed by the demonstration of Marshall *et al.* (53) and Maier & Riley (54) that sulfonamide activity against malaria can be antagonised by *p*-aminobenzoic acid. This clearly suggested basic similarity between the mechanisms of antimalarial and of antibacterial action of sulfonamides, and it therefore provided solid ground for faith in the chemotherapy of malaria by blocking nutrients with structurally similar substances. However, progress in this direction was delayed until suitable techniques were developed for cultivating malaria parasites *in vitro*. Ball, Geiman, Anfinsen *et al.* (28, 29, 30) have now described methods for obtaining growth and multiplication of *Plasmodium knowlesi in vitro*. They claim that *p*-aminobenzoic acid is one of the

chief nutrients of *P. knowlesi* under these conditions, a fact which would be in line with the exceptional potency of sulfanilamide against infections by this particular parasite in monkeys (55). However, the lead to a new antimalarial, on these lines of reasoning, was provided by the earlier work of Trager (56), working with *P. lophurae*. Although Trager was unable to demonstrate actual multiplication of the parasites *in vitro*, he did maintain them for several weeks, at body temperature, and he then showed that calcium pantothenate favours such survival. This led to investigation of a number of pantothenic acid analogs, and antimalarial activity was found in *d*-pantoyltaurylamide by Mead *et al.* (57) and in pantoyltauramido-4-chlorobenzene and other analogs by Brackett *et al.* (58). The most promising of this group was phenyl pantothenone (59) which had been described by Woolley (60, 61) as a competitive antagonist of pantothenic acid. The degree of activity in *P. gallinaceum* and in *P. lophurae* infections in chicks is of about the same order as that of quinine, but activity against *P. vivax* in man has proved to be but slight (49). Details of the exploration of this chemical series illustrate well some of the typical difficulties inherent in the search for antimalarial compounds. The initial lead was provided by Trager's *in vitro* observations on *P. lophurae* (56), but when compounds of the series were then tested on ducks infected with this parasite, no antimalarial activity was encountered, and the series might well have been abandoned, had experience not dictated the necessity of screening as many compounds as possible on several species of parasite and in several species of host (59). Marshall (59) and Davey (62, 63) quote striking examples of the frequently encountered complete lack of correspondence between various avian and human infections, in regard to the prophylactic or curative activity of particular antimalarial compounds.

Identifying and aiming at a specific metabolite is, of course, invaluable for the development of biochemical and chemotherapeutic theory, and is always capable of leading, tangentially if not directly, to important new chemotherapeutic agents. It is, however, perhaps too exacting as a main line of attack in the search for such agents, in the present state of our knowledge. It constricts the target area to an exceedingly fine point within the wider and eventual objective, the parasite cell, and it is likely that chemotherapeutic drugs of real value in clinical practice will, for some time to come, continue to be discovered by what may be called inspired opportunism, aided and to some extent guided by deductive rather

than by inductive reasoning. Our understanding of the details which underlie modes of drug action is still too imperfect, and will, for the present, no doubt continue to trail behind, rather than lead inevitably and directly towards the introduction of really useful chemotherapeutic agents. This is not to say that due attention to modern knowledge of modes of drug action is unlikely to contribute anything to the discovery of such compounds. Results of the utmost practical value may emerge whilst this knowledge is kept well to the fore, but they may not be reached along the lines originally anticipated by theory. This may be illustrated by the researches which led to the discovery by Curd, Davey & Rose (64, 65) of the antimalarial drug, "paludrine," later examined intensively in man by Adams *et al.* (66), Maegraith *et al.* (67), Fairley *et al.* (68) and others. In the earliest stages of this work it was decided to explore the antimalarial possibilities of derivatives of pyrimidine. One (but by no means the only) reason for selecting this heterocyclic type was the fact that it is an integral part of a number of essential cellular constituents, such as for example nucleoproteins, the chief components of the cellular nucleus. Conceivably therefore, it was argued, pyrimidine derivatives might exercise an antagonising effect on some essential growth factor for the malaria parasite. The hypothesis appeared to receive considerable support not only from the fact that a structural resemblance could be pictured between riboflavin and certain pyrimidine derivatives which were shown to exhibit antimalarial properties, but that these (and other) antimalarial substances were in fact found by Madinaveitia (69) to act as riboflavin antagonists in regard to *Lactobacillus casei*. As their work developed, Curd, Davey & Rose came to the conclusion that antimalarial activity might be associated with the linking of an aryl and an aminoalkyl group to the pyrimidine nucleus through groupings capable of prototropic change. This led to the question whether the function of the pyrimidine ring might be no more than to provide two nitrogen atoms suitably oriented to permit the required prototropy. In this event the cyclic feature of the pyrimidine ring would not be necessary. This was, in fact, found to be the case, and the work thus led to "paludrine," a phenylalkylbiguanide.



Paludrine

This compound does not exhibit the feature of riboflavin antagonism, demonstrated by Madinaveitia in the pyrimidine prototypes, and Curd & Rose (70) now suggest the possibility that its antimalarial activity may depend on interference with the porphyrin metabolism or enzyme systems of the parasite. This is postulated on the grounds that the compound forms a chelated copper complex, which bears close structural resemblances to natural porphyrins. The work has therefore led far away from the point where pyrimidine compounds were selected partly on the basis that they might interfere with structurally related essential growth factors of the parasite, and it has in a sense turned full circle, in that a favoured hypothesis as to the mode of action of the series is again interference with a substance essential to the parasite, by the agency of a structurally similar molecular complex.

Needless to say, work of this nature cannot dispense with the necessity of synthesising and testing considerable numbers of chemical variants of each type, however capably the general course of the work may be steered by rational considerations. There are innumerable instances of considerable differences of chemotherapeutic activity associated with very slight differences in chemical structure. As a recent example, Lutz *et al.* (71) report a thirty-two-fold difference in activity against *P. lophurae* infections, between α-di-*iso*butylaminomethyl-7-chloro-2-*p*-chlorophenyl-8-methyl-4-quinolinemethanol and its di-*n*-butylamino isomer.

The work of Hellerman *et al.* (72) on the inhibition of *d*-amino acid oxidase activity gave little support to the idea that the antimalarial activity of quinacrine is dependent on its structural resemblance to riboflavin or other isoalloxazine derivatives. Unrelated substances, such as quinine, pamaquin, nonantimalarial quinolines, and sulfonamides shared with quinacrine an antagonistic effect on flavine-adenine-dinucleotide, as observed by the behaviour of isolated enzyme preparations, although studies on rate-concentration relationships, showed that the antagonism of quinine differed from that of quinacrine in being of a reversible type. This illustrates that competitive inhibition of an enzyme reaction is not necessarily dependent upon close structural resemblance between inhibitor and prosthetic group.

Resonance.—Mention should here be made of one further feature which entered into the considerations of teams recently engaged in the search for new antimalarials. In connection with quinacrine and pamaquin, Schönhöfer (73) had drawn attention to the

association of antimalarial activity with the possibility of prototropy, which would result in a system of quinoid linkages between the nitrogen atoms of the ring system and of the alkylamino side chain, leading perhaps to oxidation in the body to a quinonimine type of compound. However, this left unexplained the established fact that the high degree of activity of quinacrine and mepacrine is also largely dependent on methoxy groups appropriately positioned in each compound (as well as in quinine). Curd, Davey & Rose (64) and Blanchard & Schmidt (48) have therefore sought to explain the potentiating influence of the methoxy group on the basis of arguments involving current theories of resonance.

The association of resonance with activity against microorganisms was also stressed by Albert (74) in studies on the basis for the antibacterial activity of acridine and triphenylmethane compounds. He wrote "it may safely be predicted that a vast number of new antiseptics will be found among such amines of high molecular weight as have highly resonant ions." The subsequent studies on acridines as antibacterial agents, by Albert, Rubbo and coworkers (75, 76) are a model of comprehensive approach, in which chemicophysical factors taken carefully into account included solubility, ionization constants, oil/water partition coefficients, adsorbability at various interfaces, absorption of light in different portions of the spectrum, reduction potentials and heats of combustion.

Types of drug antagonism.—For recent discussions and classifications of antagonism the reader is referred to Gaddum (77), McIlwain (78), and to an excellent and exhaustive review by Roblin (79).

What may be regarded as a special application of antagonism is the varying degree of antibacterial or antitrypanosomal activity dependent on changes in pH of the environment. Basic substances, in general, become less active with rise in hydrogen ion concentration, e.g., acridines (80), streptomycin (81); correspondingly, acidic substances become more active under these conditions, e.g., acid-substituted arsenoxides (82), penicillin (81). This has been interpreted quite simply, especially by Albert and by Abraham & Duthie as being due mainly to competition between H^+ and the cation of the basic substance, or between OH^- and the anion of the acidic substance, for fixation on the bacterial cell, so that, in a sense H^+ or OH^-, respectively, may be regarded as antagonising

or interfering with antibacterial (or trypanocidal) activity of the compounds concerned. An alternative interpretation in such cases, (strongly advocated by Eagle in regard to the trypanocidal activity of acid-substituted arsenoxides) is that the undissociated acid or base is more active than the ionic form, perhaps because it more easily penetrates into the cell. Increase in hydrogen ion concentration would therefore favour the activity of acidic compounds, since the concentration of undissociated acid would be increased, whilst it would hinder the activity of basic compounds, since the concentration of undissociated base would be decreased. Studies on sulfonamides from this point of view have led to important conclusions. The fact that sulfanilamide becomes more active with decrease in hydrogen ion concentration has been interpreted as evidence that the anionic state is one of the main determinants of sulfonamide activity (83, 84). This appears at variance with the general finding of decrease in activity of anionic substances with decrease in hydrogen ion concentration. Bell & Roblin (85), pursuing the matter further, plotted the pK_a of a large series of N^1-substituted sulfanilamide derivatives against their antibacterial potency *in vitro*, and they found that activity did not increase uninterruptedly with increase in acid strength, but reached a maximum at pK_a values of 6 to 8, and then began to fall. They concluded that another determining factor for antibacterial activity, apart from dissociation of the molecule, is the electron attracting power of the N^1-substituent, which may be correlated directly with antibacterial potency.

A class of compound which has been shown to be capable of antagonising many examples of antibacterial or trypanocidal action is that characterised by the presence of the -SH group. In some cases, as in the trypanocidal action of arsenicals [Voegtlin *et al.* (86)] or the antibacterial action of mercurials [Fildes (87)], the antagonism is clearly related to the well-substantiated high degree of affinity between drug and -SH, and is sufficient to provide the basis for explaining the mode of action of these particular drugs by an inactivation of essential -SH groups in the parasite cell. In other cases, as for example in the antagonism of glutathione towards the antibacterial action of quinacrine, described by Silverman & Evans (88), the affinity is very considerably less, and cannot contribute much to an understanding of the mode of action of the compounds concerned. Penicillin and various other antibiot-

ics have been shown to be inactivated by -SH compounds (89 to 93), and the mode of action of these antibiotics has been conceived as possibly dependent on the inactivation of -SH groups in the bacterial cell. Such explanations do not, of course, necessarily follow in a straightforward manner from the mere demonstration of antagonism of drug action *in vitro* by -SH compounds. Colwell & McCall (94) found that although 2-methyl-1:4-naphthoquinone and its 3-methoxy derivative both exhibit antibacterial properties, the former is antagonised by -SH compounds, whilst the latter is not. It is, however, surely likely that these two compounds possess a similar mode of antibacterial action, rather than that the mechanism should depend in the one case but not in the other on an inactivation of -SH groups of the bacterial cell.

As indicated above, antagonism or interference with chemotherapeutic action of a particular drug does not inevitably imply that the mode of action of that drug is by inactivation of chemical groups, essential for the parasite, which are similar to chemical groups of the antagonising or interfering substance. Thus, Williamson & Lourie (95) have demonstrated interference with the trypanocidal action of γ-(*p*-arsenosophenyl)butyric acid by *p*-aminobenzoic acid. However, the former substance shares with other arsenoxides a liability to inhibition of its trypanocidal activity by glutathione. It was tentatively concluded therefore that the mode of trypanocidal action of *p*-arsenosophenylbutyric acid is essentially the same as that generally accepted for the other arsenoxides, namely by inactivating -SH groups of the trypanosome, and that the interfering effect of *p*-aminobenzoic acid, in this case, may be due merely to its preventing or limiting admission of the arsenical into or onto the trypanosome cell.

Whether or not every instance of antagonism by -SH groups points to inactivation of such groups as the essential mode of action of the particular drug under consideration, there can however be little doubt that -SH groups of the parasite are among the most vulnerable components of the cell. They provide a broader target than any individual metabolite in that they are widely distributed, being essential for the activity of many important enzymes, such as, for example, glyoxalase, succinic acid dehydrogenase, triosephosphate dehydrogenase, and certain pyruvate oxidases. Consideration of the reactivity of -SH groups was an important feature of the wartime investigations on methods of combating chemical

warfare agents, summarised by Peters *et al.* (96), Waters & Stock (97) and Dixon & Needham (98). 1:2-Dithiols were shown to be much more effective than monothiols in reversing the inhibitory effects of arsenic on enzyme systems which depend on -SH groups, and this led to the discovery of *BAL*, or British Anti-Lewisite (i.e., 2:3-dimercaptopropanol), as a powerful antidote to poisoning by arsenic and other metals. The detoxicating effect against arsenic is due to speedy formation of a rapidly excreted cyclic compound, by reaction of the vicinal -SH groups of the dithiol with the toxic metal. However, *BAL* is itself not without toxic properties, and Danielli *et al.* (3) thereupon showed, on a sound rationale, that its toxicity could be very much diminished by use of its *O*-glucoside, known as *BAL-intrav*. Although the use of *BAL-intrav* does not come strictly within the generally accepted connotation of chemotherapy, for the reason that it would not be directed against an infection, its development is mentioned here as a model of neat and concise reasoning through which a means was devised of combating a compound's toxic effects—and the limitation of toxicity to the host is, of course, very much a concern of chemotherapy.

The Host-Parasite-Drug Complex

Emphasis upon the reactions which occur between drug and parasite (as in studies *in vitro*) tends perhaps to neglect the fact that chemotherapy is ultimately concerned with the triple relationships of drug, parasite and host. The complex result of this relationship can only be adequately studied on what may be termed composite as well as on analytical lines. That is to say, whilst it is necessary for the components of the triple integration to be studied separately—drug in relation to host (i.e., toxicology), parasite in relation to host (i.e., parasitology), and drug in relation to parasite—attention must then (or concurrently) be directed to the modifications introduced when the three factors of drug, parasite and host are all thrown together.

Thus, *in vitro* studies may, on occasion, give an invaluable lead to new chemotherapeutic agents. When, however, the drug is introduced into the host, the entire picture may be radically modified through factors of absorption, distribution, excretion and degradation which immediately come into operation, and which cannot all be reproduced *in vitro*. Apart from any other considera-

tion, the drug, or its active degradation product, must reach the actual site of parasitisation. For example, a trypanocidal agent, no matter how potent *in vitro* or in the circulating blood, will be useless to the advanced case of sleeping sickness if it is unable to penetrate to the trypanosomes in the central nervous system (99). Studies on the distribution of chemotherapeutic compounds within the treated animal are thus of prime importance, and techniques of treatment are constantly shaped by the necessity of bringing the drug or its active principle right to the parasite. As a simple example, the many expedients described (100, 101) for preventing destruction of penicillin in the intestinal tract generally have as their real objective that oral treatment may result in effective concentrations of penicillin in the blood. Local treatment would appear to provide the readiest means of bringing drug into juxtaposition with parasite in wounds, or in infections of surfaces or cavities. There may however be considerable difficulty in reaching deep loculi or sinuses, and numerous other problems and complications arise in connection with local chemotherapy (102). Florey *et al.* (103) have investigated the relative advantages of systemic and local treatment of wounds by penicillin. Among other findings of more practical importance, they found that after an intramuscular injection penicillin can usually be detected in wound-exudates for many hours longer than in the blood. They suggest, accordingly that if the presence of penicillin in wound exudates indicates its presence also in inflammatory exudates, this would go towards explaining the good results claimed for the use of penicillin injections at widely spaced intervals in the treatment of early syphilis (104, 105), gonorrhoea (106) and acute inflammatory conditions (107).

Sulfonamide activity against intestinal infections.—In the search for new chemotherapeutic agents intended against a particular disease of man, absorption, distribution, degradation, and many other factors—usually even the species of parasite—will necessarily differ considerably in the test animal from those in man, and the limitations thereby imposed must constantly be taken into account in the employment of screening techniques. In the search for antimalarials, as indicated above, the difficulties may be particularly acute. In regard to sulfonamides intended for intestinal infections, Poth (108) has reviewed the steps by which the virtues of sulfasuxidine and sulfathalidine were first brought to light. The

ordinary procedure of depending upon survival-rates in mice treated after intraperitoneal inoculation of the test organism was considered unsuitable; dogs were chosen because their normal intestinal flora is similar to those of man, and the crucial screening test therefore consisted of observations on the changes in the numbers of intestinal coliform organisms produced by oral treatment of these animals.

Moore *et al.* (109) provide an interesting example of an investigation in which *in vitro* studies of drug on parasite, and studies of drug on host, are integrated so that judgment may be formed as to which particular member of a series of alternative compounds is likely to be the best. Four sulfonamides were investigated, namely, the two freely absorbed compounds sulfathiazole and sulfadiazine, and the poorly absorbed sulfaguanidine and sulfasuxidine. It was found, in general confirmation of previous workers, that sulfathiazole is about 2.5 times as active as sulfadiazine, forty times as active as sulfaguanidine, and one hundred times as active as sulfasuxidine on various shigella and salmonella strains *in vitro*. Sulfathiazole may be brought into the large bowel (*a*) by failure of absorption, or by excretion into the bowel, after oral administration; (*b*) by gradual hydrolysis of sulfasuxidine (i.e., succinyl sulfathiazole) or of sulfathalidine (i.e., phthalyl sulfathiazole); or (*c*) by administering enteric-coated sulfathiazole, so that the compound is not liberated until the dose reaches the large bowel. The authors made determinations of urinary and faecal sulfonamide, after various forms of treatment in normal volunteers and in enteric patients. They found, for example, that although sulfathiazole is substantially more active than sulfadiazine *in vitro*, this advantage is more than counterbalanced by the fact that sulfadiazine attains a higher concentration in the faeces than does sulfathiazole, after doses of comparable size. Uncoated sulfadiazine was therefore judged likely to be slightly superior to sulfathiazole in practice. Considerations of this type led to the suggestion that the following might be the order of preference of the preparations tested: Enteric-coated sulfadiazine, similarly coated sulfathiazole, sulfasuxidine, uncoated sulfadiazine, uncoated sulfathiazole, and sulfaguanidine. Obviously, much will depend on the efficiency of the coating used. It is, of course, practically impossible in such an investigation to take into account all the imponderables which contribute to the eventual therapeutic result, so long as their relative

importance remains unknown. Thus, while it seems rational to hold that a high sulfonamide concentration should be produced within the large gut, it is by no means certain that low blood, high faeces levels are necessarily more effective in various forms of bacillary dysentery than are high blood, low faeces levels. Early in the war in the Middle East, and later in New Guinea, sulfaguanidine had been used for bacillary dysentery with remarkably favourable results, as described by Fairley & Boyd (110, 111). It seemed reasonable to attribute its success largely to the fact that it is relatively poorly absorbed, so that its full effect is exercised within the lumen of the bowel. The unique value of poorly absorbed sulfonamides in bacillary dysentery was however subsequently challenged by a number of workers, e.g., Hardy & Watt (112) and Scadding (113), and it became clear that excellent results could be obtained with freely absorbed compounds, such as sulfathiazole and sulfadiazine. In fact the United States War Department, in 1944, declared sulfadiazine to be the drug of choice in the treatment of bacillary dysentery (114). Other freely absorbed sulfonamides may prove to be equally efficacious.

Chemotherapy and Demography

The great strides made by chemotherapy, in recent years, towards the potential control of disease on a mass scale has been paralleled by considerable technical progress in certain other means towards the same end. There is, for example, the discovery of DDT and other insecticides of unprecedented potency. Such advances must surely contribute much towards the demographic trends which are already clearly apparent. Mankind is faced with staggering increases in numbers within the next few generations. These increases will be of a differential nature, favouring in particular the Asiatic peoples, and are more than likely to give rise, in due course, to fearful stresses both within and beyond Asia. Thompson (115) describes how the population changes produced by increasing industrialisation, higher standards of living, better sanitation, and improved internal communications, apparently assume the following pattern. Death rates soon begin to decline, but without a concomitant initial falling-off in birth rates. There is, therefore, a more or less prolonged period during which the population increases considerably, before it eventually reaches a stationary or decreasing phase, with the onset of declining birth-

rates. Western European peoples entered the initial period—a greatly expanding population—with the Industrial Revolution. In 1800 there were rather less than two hundred million Europeans, and great outlets were available in the Americas, Australia, New Zealand and South Africa. Now, however, people of Western European stock, alike in Western Europe and in the distant lands to which they have emigrated, are in the period of relatively stationary or declining numbers, at the very time when the Asiatic peoples, in so far as it is possible to generalise about such heterogeneous groups, are to be regarded as entering the initial, expanding, phase. There are now more than one thousand million people in South and East Asia (i.e., more than five times the number of Europeans there were in 1800), on the threshold of vast increases in number, and, as Thompson writes, "there are no more Americas to be settled." In such a short period as from 1921 to 1942 the population of India, for example, increased by about 83 millions, a figure which is about two-thirds of the *total* population of the United States. By 1981 India's population is likely to have grown from the 306 millions of 1921, to about 680 millions. Again, China's population is likely to increase almost immediately, from the present total of about four hundred million, at a rate of about forty to sixty million (i.e., by a figure equal to about the total present population of England or France) per decade. Whether these colossal increases will in fact arise will not depend upon changes in birth rates, since these are expected to remain at a fairly constant level, but upon the extent to which death rates will be held in check, and these, in turn, will depend upon political stability, prevention of famine, and control of disease. And it is in the control of disease that recent and impending advances in chemotherapy (together with sanitary science) obviously play their part. The Lancet (116) has discussed the significance to the world at large of the fact that we now have powerful and potentially cheap remedies against the widespread scourges of malaria, pneumonia, and gonorrhoea (the last-named being one of the primary causes of low birth rates, by causing sterility in both sexes). Equally satisfactory means may well be discovered, before long, for dealing with other major infections, such as virus diseases and tuberculosis.

Without doubt, chemotherapy will therefore play an increasingly large part not only in the relief of suffering but also in the aggravation of some of mankind's most serious problems, for

pressure on the world's nutritional resources must vastly increase, with the impending vast increases of population. But besides contributing towards the creation of this problem, chemotherapy may also contribute largely towards its solution. One way in which this is true is as follows. The chemotherapy of diseases of domestic stock is, in some respects, far more important to mankind than the chemotherapy of disease in man himself. In Africa, for example, Bax (117) quotes that an area of four and one-half million square miles is infested with the tsetse fly. In Tanganyika alone two-thirds to three-quarters of the territory is thus affected. These areas are accordingly unfit for human settlement, on account of trypanosomiasis of man and domestic animals, and the zones which are habitable tend to become impoverished through overcultivation and soil erosion. Chemotherapy is, of course, only one and obviously not the most radical—weapon in campaigns for eliminating trypanosomiasis, but its potentialities are undoubted. Against human trypanosomiasis fairly adequate chemotherapeutic agents have been known for many years. Against *Trypanosoma congolense*, the most important trypanosome of domestic stock, however, this has not been the case, and the introduction of the phenanthridinium compounds by Browning *et al.* (118) was therefore a considerable advance. The most hopeful member of this series, so far, appears to be 2:7-diamino-9-phenyl-10-methyl phenanthridinium bromide (119, 120).

In summary of this section, then, chemotherapy, like so many of the technical triumphs of civilisation, is only ostensibly and superficially an unalloyed blessing. While solving some of our worst problems, it helps to create new ones. We may express faith in the belief that the good far outstrips the potential evil, but it is for the sociologists and philosophers to judge the extent to which this really is the case.

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DEPARTMENT OF CHEMOTHERAPY
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IMMUNOCHEMISTRY

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Several reviews have appeared (1, 2, 3, 4) covering the literature on immunochemistry since the publication of the second edition of Landsteiner's comprehensive and classic monograph (5). In view of these the range of this review has been restricted to several groups of papers concerned with central themes which it is felt are of some general interest.

A portion of the field of immunochemistry appears to be entering a new phase of its development—a period of exploration with more effective techniques of handling problems which had attracted investigators in the past. In the history of efforts to understand and control the agents of disease we may distinguish first a phase of biological exploration, marked by many distinguished investigations in experimental bacteriology and medicine. The subject then entered a period in which the chemical morphology became of prime importance. Although characterized by investigations of great fundamental biochemical interest, this phase could contribute relatively little to an immediate understanding of the mechanism of disease. Nor is this phase entirely an end; in fact, our knowledge of the basic composition of many materials of interest for disease and host resistance is but fragmentary at best. However, there are increasing signs that with the biochemical groundwork of the composition of the materials at hand, and adequate tools for investigating their interaction developed, studies can be made of the operation of these factors directly in living systems. That these studies will result in an increasing fertilization of the basic field of immunochemistry with the techniques of other related fields is obvious. In particular, contributions may be expected from enzyme chemistry, antibiotic research, genetics, bacterial physiology and nutrition. The basic methods of protein and carbohydrate chemistry have, of course, always formed a part of the immunochemist's armamentarium.

Methods

The Tiseleus electrophoretic technique is finding increasing use as a pilot procedure in investigating biological materials. The

monograph of Svensson (6) presents a full account of recent developments in the theory and technique of this method. Longsworth (7) has discussed the practical problems connected with the installation and adjustment of the apparatus. Zittle & Harris (8), and Zittle (9) have shown that the addition of borate may greatly facilitate the separation of polysaccharides by electrophoresis or by precipitation methods. A general review on fractionation methods of polymeric substances has appeared (10). Falconer & Taylor (11) have presented a specific-property solubility test for protein homogeneity. The lyophile process (12, 13) has come into general laboratory use for the drying of proteins and polysaccharides. Stanley (14) has described the advantages of the Sharples centrifuge for certain types of virus preparations. Ultrasonic techniques have been used for releasing bacterial enzymes (15). Antigens have been released by the use of ether (16), or heat (17). The use of protein films in immunological studies has been reviewed by Rothen (18).

Bacterial Nutrition

Our knowledge of the factors necessary for the growth of microorganisms has increased termendously during the past decade (19,20). Synthetic media have an appreciable advantage for the growth of microorganisms necessary for immunochemical work in that they are of uniform and known composition, and lack the preformed serologically active polysaccharides often present in peptone (21) and agar (22) media. However, relatively few such investigations employing synthetic media have been reported, due largely to the low yields so frequently obtained. Nevertheless with continued improvement of these media to the point where they give optimum yields it may be expected that their use for immunochemical investigations will increase. As was pointed out by Knight (23) some time ago a certain amount of caution will have to be exercised to ascertain that the desired serological and other biological properties of the organisms are retained during growth in such media. As an example, it has been found in unpublished work in the reviewer's laboratory that a strain of *Shigella dysenteriae* grows well on several synthetic media but becomes inagglutinable after a few passages. Further work is in progress to determine whether this is the result of a failure to produce the antigen or a masking process due to the production of an additional anti-

gen. The most direct evidence for differences in metabolic products as a result of factors present in the media has come from studies on toxin production (see below). More important perhaps than differences resulting from growth in synthetic *vs.* the classical culture media are differences between the metabolic activities of microorganisms growing *in vivo*, and in any available culture medium. There are a number of diseases, such as pneumococcus pneumonia, in which all of the signs and symptoms cannot be accounted for on the basis of products derived from the organisms grown on the usual media. It is not certain, but perfectly possible, that some of these unexplained factors may be due to products of the organism not produced in the usual media. While immunochemical work has been done on microbial products obtained from *in vivo* growth, in spite of the greater complexity of the accompanying material, there is every hope that simplifications can be effected by reproducing the essential features of the *in vivo* growth in synthetic culture medium. In some instances this can be accomplished by relatively small changes. An illustration is described below in the discussion on anthrax.

Bacterial Toxins

The past few years have resulted in a considerable expansion of our knowledge of bacterial toxins. Two of them have been crystallized. Others have been obtained in more potent form. Of the greatest interest are the recent studies of Pappenheimer (24) on diphtheria toxin, for here we have the first indication of the role which an important serologically active substance may play in the metabolism of the cell producing it. In addition, these studies may serve to elucidate the mechanism by which diphtheria and perhaps certain other toxins produce their pathological effects in susceptible hosts. The starting point was the now well-recognized observation (25) that the amount of free toxin produced by *C. diphtheriae* is a function of the amount of iron contained in the culture medium. Quantitative measurements (24) showed that at the concentration of iron optimum for toxin production growth of the organism was good but not the maximum possible. If the amount of iron was increased growth also increased; however under these conditions toxin production decreased proportionally. Simultaneous measurements of the amount of porphyrin contained in the organisms also showed a linear decrease with excess iron, the relations being such

that for every four atóms of iron added to the culture medium over the amount optimum for toxin production, four molecules of porphyrin and one molecule of toxin disappeared.

Although growth of the organism is depressed by a deficiency of iron in the medium it continues nevertheless to synthesize porphyrin and toxin in the molecular ratio four to one. Since the iron necessary for completing the complex is not present, the two do not combine and free toxin escapes into the medium. When sufficient iron is present, it is taken up by the cells and the toxin combined with porphyrin. The end result is therefore a diminution in the amount of free toxin in the medium. From these results, Pappenheimer has concluded that diphtheria toxin is the protein moiety of an iron-containing respiratory enzyme present in *C. diphtheriae*. The obvious inference that the detrimental action of diphtheria toxin in humans and animals depends on its interference with a metal-containing enzyme of the host is now being investigated.

Studies on the toxin of *Cl. botulinum*, type A, an agent of food poisoning, have not yet yielded the theoretical implications of the above but have served to characterize chemically and physically one of the most potent poisons known (26). The material, prepared from cultures grown in a casein-corn steep liquor medium, is subjected to a number of fractionation steps with ammonium sulfate and other agents. One important step involves shaking with chloroform, during which the potency of the medium increases from 800,000 mouse M.L.D. per ml. to 15–30,000,000 M.L.D. per ml. The mechanism of this increase is obviously of some interest. The toxin is finally isolated in the form of needle-shaped crystals. Both the amorphous and the crystalline material had quite similar chemical properties, and were homogeneous by electrophoresis. The nitrogen content of 14.3 per cent conforms to that of a protein. For the amorphous preparation the M.L.D. per mg. of nitrogen was 120.7×10^6. The L D_{50} per mg. of nitrogen is given as 198.5×10^6 for the amorphous material and 239.9×10^6 for the crystalline form.

Simultaneously with the above there appeared an independent announcement (27) by Abrams, Kegeles, & Hottle of the successful isolation of crystalline Botulinus toxin, type A. The purification procedure consisted of a number of steps involving precipitation with sodium sulfate or acid. The crystalline material was homogeneous by electrophoresis and gave a single ultraviolet absorption

band at 278 mμ. These two properties were found useful in following the separations. The nitrogen content of the crystalline material was determined as 14.1 per cent, and the isoelectric point as 5.6. Twice recrystallized toxin contained 220×10^6 mouse M.L.D. per mg. of nitrogen. More detailed physical measurements (28) indicated a single symmetrical boundary in the ultracentrifuge, with an S of 17.3. The diffusion data fitted the ideal curve. The molecular weight calculated from these two properties is 900,000, compared with value of 1,130,000 from diffusion and viscosity (29). The amino acid analyses indicate that it is a typical protein (30).

Similar characterizations have also been made for tetanus toxin by Pillemer, Wittler, & Grossberg (31, 32), who have shown that the toxin can be isolated from culture filtrates of *Cl. tetani* by means of methanol precipitations under carefully controlled conditions. Crystals of the toxin were secured which contained 3,500–4,000 Lf units and 50 to 75×10^6 mouse M.L.D. per mg. of nitrogen.

Recent studies (33) on the biological effects in mice of crystalline tetanal toxin are not in accord with the commonly accepted idea that the incubation time in tetanus intoxication cannot be reduced below eight hours, no matter how large a dose is given. The extreme potency of the crystalline material permitted the injection into a single animal of amounts as high as 500,000 M.L.D. This dose, corresponding to 6.4μg. toxin N resulted in symptoms as early as thirty-five minutes, and in death in one hour. The toxin N corresponding to 1 M.L.D. was found to be 0.000013 μg. The preliminary conclusion was advanced that tetanal toxin is only an intermediate agent in the production of tetanus and that another substance formed in the host is the actual toxic agent.

The development of more active preparations of the so-called Shiga exoneurotoxin has been reported by Dubos & Geiger (34). This material appears to be a protein and is distinct from the somatic enterotoxin, which is attached to the type-specific polysaccharide complex. Although present in the smooth cultural forms, it is more easily prepared from the rough variants since no isolation from the toxic polysaccharide need then be made. As in the case of diphtheria toxin the yield was found to be influenced markedly by the content of iron in the medium, and to be increased greatly by vigorous oxygenation of the latter. The most active preparations possessed an L D_{50} of 1 to 10 μg. for mice and rabbits. They were readily detoxified by 0.5 per cent formalin at pH 8.5, and the re-

sulting toxoid was found to be antigenic in mice. A similar detoxification of autolysates of *S. dysenteriae* by ultra violet irradiation has been reported by Branham & Habel (35). It has been reported by Treffers (36) that the toxic somatic polysaccharide-protein complexes of *S. dysenteriae* and *E. typhosa* can be detoxified by acetylation without losing protective antigenicity.

Tagged Elements in the Study of Bacterial and Virus Antigens

Tracer studies employing artificial radioactivity have been made in many fields of biology, and with the increasing availability of such materials applications to immunochemistry will undoubtedly become even more common. Ross (91) has carried out studies with radiophosphorus, P^{32}, which was incorporated into streptococci through the culture medium and shown to be present in various fractions of the organism, including the M-antigen released by supersonic disintegration. The specific activity of phosphorus in the bacterial extracts was found to be only one-half of that in the culture, which would indicate some selective metabolism of ordinary phosphorus in preference to radioactive phosphorus. Stanley (92) showed that radioactive phosphorus could be incorporated into tobacco mosaic virus nucleoprotein. Further studies along these lines have been made recently by Libby & Madison (93). After injection of the virus tagged with radioactive phosphorus into mice, the radioactivity could be detected in the liver and spleen, and later in the urine and feces. It could also be shown that breakdown of at least the nucleic acid portion of the virus had occurred in the tissues by the demonstration of the incorporation of P^{32} in nonvirus lipid phosphorus of the tissues. The constancy of the specific activity of the preparations on repeated recrystallizations provides some evidence for the association of the radio phosphorus with the virus which it was desired to follow. The need for establishing this point as rigidly as possible is evident from an investigation of Dr. P. M. Freire and the reviewer which is now in progress. Radioactive phosphorus has been incorporated into Shiga dysentery organisms through the culture medium, and preparations of the soluble polysaccharide antigen have been obtained of the same specific radio activities as that of the culture medium and of the whole organisms. The phosphorus content remained constant through a number of precipitations with various solvents, a

criterion usually taken as evidence of homogeneity for polysaccharide preparations. If the antigen were treated with its specific antibody it could, however, be shown that only one-sixth to one-ninth of the radiophosphorus could be detected in the specific precipitates. It is thus apparent that most of the phosphorus in these preparations is not directly associated with the polysaccharide antigen, although it is not readily separated by the solvent fractionation procedures adopted. It may be desirable to utilize, wherever possible, specific biological properties such as these to establish for tracer antigens at least their "radioactive homogeneity," since it is obvious that quite misleading results could be obtained in metabolic studies if the radioactivity did not follow to a large extent the material which it is desired to trace. Precipitation of radioactive elements with antiserum may also prove to be a sensitive general method for establishing the true connection of particular elements with a given substance. Although this is but a variation of the criterion (45) discussed below in connection with the blood group A substance, it is a significant modification since it may not be possible to make direct chemical estimations of non-radioactive elements present in a small percentage under conditions in which they are used as antigens, particularly against sera containing large amounts of the element in question. It would be of some interest to reexamine by this technique a number of the polysaccharides for which, for example, phosphorus has been reported to be a definite constituent.

Bacterial and Blood Group Antigens

Two general reviews on serologically active polysaccharides have appeared (37, 38). While a great deal of chemical information has accumulated on the properties of polysaccharides from various natural sources the criteria of homogeneity adopted have not been as rigid in most instances as those generally deemed necessary for protein investigation (39, 39a). In the majority of cases the only criterion advanced has been the constancy of one or more analytical properties after repeated attempts at fractionation with one or more solvents. In a few instances we have little more than the author's word as to the purity of his material. As all who have worked in this field are aware, however, high molecular weight polysaccharides offer difficulties not presented even by such complex substances as proteins. From the meagre data available it may

be surmised that many polysaccharide preparations of homogenous chemical composition still consist of a dispersion of molecular sizes. How much of this is due to the method of preparation cannot be stated, but it is quite possible that even in the organism polysaccharides occur in various molecular states. Due to various technical difficulties few sedimentation studies have been made in the ultra centrifuge (40). As Kraemer has pointed out (41), too, the sedimentation constants of long linear molecules are not very sensitive functions of relative chain lengths, so that the homogeneity indicated by the single-peaked sedimentation diagram may be more apparent than real. A number of diffusion determinations have been made, (42, 43), but it has been noted that preparations of glycogen which are known to be polydisperse may give ideal normal diffusion curves (44). As far as the reviewer is aware no attempt has been made to apply phase rule solubility determinations as a criterion of polysaccharide homogeneity, nor is it quite clear what degrees of polydispersities could be detected by such methods.

The development of new criteria of homogeneity which are applicable to polysaccharides would therefore be most welcome. Several recent studies illustrate this point. It has been known for some time that the activity of the blood group A substance from hog gastric mucin varied with different batches and that some hogs failed to produce material of group A specificity. In an investigation of this, Bendlich, Kabat, & Bezer (45) found that all hog mucin yielded polysaccharide-protein complexes but the material from some animals was inactive while others of the same chemical composition possessed group A serological activity. The possibility then occurred that the activity even in the most active preparations represented a minor and undeterminable fraction of the total chemical substance. To resolve this point these investigators treated active preparations with anti-A antibody and estimated by means of glucosamine determinations the proportion of the added polysaccharide which was specifically precipitated. After correcting for the glucosamine contained in the antibody protein it was found that 84 per cent of the glucosamine of the active antigen preparations had been precipitated. Since the polysaccharides contained 33 per cent of glucosamine it was evident that at least $33 \times 84 = 28$ per cent of the total antigen had been involved in this specific biological reaction, and if the original test assumption that

the active and inactive materials were of dissimilar composition were true this difference should have been detectable in the overall composition. Since it was not, and also since there was no evidence that the glucosamine is not an integral part of the polysaccharide molecule it must be concluded that the material is at least largely homogeneous and that the differences in activity are due to differences in arrangements of the same components rather than to differences in composition. This criterion of homogeneity is based on the principle that in the region of antibody excess all of a homogeneous antigen is precipitated, a premise which has been verified in a number of other instances in which antigenic homogeneity was less at issue (46, 47). A somewhat similar criterion, useful for detecting mixed antigens by means of clear-cut equivalence zones in antigen-antibody reactions, had been proposed previously (48, 49, 50). In its general applicability the foregoing method is of course not limited to "tagging" the antigen by means of glucosamine analyses; any suitable analytical marker will serve. A further application was briefly noted in the discussion of radioactive tracers above. Another use of the specific serological behavior of polysaccharides to establish at least their antigenic homogeneity has been made by Perlman & Goebel (51). A considerable number of serological types of Flexner dysentery bacilli are known. One contains an antigen designated as I, another antigen III, and a third contains the reactivities of both I and III. It had long been postulated that this type I-III antigen consisted of a mixture of the two distinct antigens. In disagreement with this, however, the above investigators found that the polysaccharide-protein antigen complex derived from type I-III organisms appeared to be chemically homogeneous since its analytical composition did not vary under various fractionation procedures. The Tiselius electrophoretic method was of little value in this instance since the mobilities of the Flexner antigens of different specificities are too close to permit analysis of differences (51). By appropriate serological analysis it could be established nevertheless that the I-III antigen behaved as a unit serological character since no fractionation could be accomplished with sera specific either for type I or type III activity.

Information as to the state of degradation of serologically active polysaccharides can often be obtained from the amounts of antibody precipated (52) and the quantitative reaction curves have been shown by Heidelberger & Kendall (53) and by Treffers,

Moore, & Heidelberger (54) to be consistent for several systems with chemical data on the type of the links split on hydrolysis. The need for caution in interpreting simple qualitative data obtained with degraded materials is evident from the results of Brown & Robinson (55) and MacPherson and her co-workers (56), who have found the volumes of serum reacting initially with degraded pneumococcus or *H. influenzae* polysaccharides to be larger than those reacting with the undegraded preparations. Appropriate quantitative procedures readily established the true situation, however (55, 56).

Further information as to the nature of the so-called T antigen of group A hemolytic streptococci has been obtained by Lancefield & Dole (57). Although antigenic in mice it does not confer protection. It was formerly thought to be a type-specific antigen but cross agglutination has been observed and it is evident that several strains may contain common T antigens. Although the T antigen is heat labile, it is quite resistent to enzymatic digestion and for preparative purposes can be released with pepsin or trypsin. The usual extractive solvents, diethylene glycol, pyridine, etc., do not release it from the cell. The T antigen is a protein, with a nitrogen content of 14.25 per cent. The crude preparations yielded three peaks on electrophoretic analysis; ultracentrifugal examination of the peak containing T activity showed it to consist of at least two components. The nucleic acid content could be reduced with ribonuclease, and is stated to be negligible in the best preparation. The type-specific agglutination of streptococci is inhibited by an excess of the soluble T-protein. In this report comparative data are also included on the chemical behavior of the M-antigen, the type specific protein which confers protective antigenicity on the organisms.

Bacterial Enzymes

Several recent studies on the properties of bacterial enzymes are of direct immunological interest and will be considered here. Streptococci grown under certain conditions do not contain M-antigen, although they may be capable of producing it if the environment is modified. Further studies by Elliott & Dole (58) have clarified this phenomenon. The loss of M-antigen is apparently due to its digestion by a proteolytic enzyme contained in the cultures. The enzyme exists normally as an inactive precursor but

the addition of small amounts of trypsin, or the enzyme itself, results in autocatalytic transformation of the precursor to the active form, which then destroys the antigen. Studies on other proteolytic enzymes of bacteria have been reviewed by Grob (59). That the alpha toxin of *Cl. welchii* is a lecithinase has been known since the work of Macfarlane & Knight (60). A study has now been made by Zamecnik & Lipmann (61) of the competition of lecithin and *Cl. welchii* antitoxin for the toxic lecithinase. The reactions were followed by manometric measurements of the carbon dioxide displaced from bicarbonate buffers by the phosphorylcholine liberated during the hydrolysis. The combination of lecithin with toxin was found to be inhibited in direct proportion to the amount of antitoxin added and could be reduced to zero with sufficient antibody. On the other hand, if the lecithin were added as little as five seconds in advance, inhibition of the combination of antitoxin with toxin could be detected. Although the enzyme only splits lecithin, phosphatdyl ethanolamine, ox brain cerebroside and sphengomyelin were found to prevent lecithin hydrolysis if they were added to the toxin first. Evans (62) has concluded from a study of the protective antibodies to *Cl. welchii* that antibodies to collagenase, hyaluronidase and the theta toxin are not nearly as important as are the antibodies to the alpha toxin.

Viruses

Only a part of the literature appearing since the last review (3) can be considered here. Knight (63) has examined the serological cross reactions of influenza virus preparations grown in eggs and in mouse lungs, and from the antibody response has obtained information on the virus obtained from human infections. Antisera to the sedimentable protein of normal chick chorioallantoic fluid were prepared in rabbits. The cross reactions as studied by the quantitative precipitin method of Heidelberger and Kendall revealed that the PR 8 and Lee virus strains removed 20.3 and 29.6 per cent, respectively, of the amount of antibody taken out by the normal chicken protein used for immunization. With excess of virus it could be shown that the PR 8 strain removed 76 per cent, and the Lee virus 86 per cent of the total antibody. The quantitative features of the reaction were somewhat unusual, which made calculation of the amount of antibody precipitated more indirect, but there appears no doubt as to the essential correctness of the general

conclusion that the greater part of the antigenic specificity of the normal chick chorioallantoic protein is also represented in the virus. Significant cross reactions were obtained with the normal chick antigen, with the Lee virus, and with PR 8 virus from mouse lung when they were tested against a rabbit antiserum to egg-grown PR 8. The PR 8 strain from mouse lung was neutralized by the antibody, and its agglutination with chicken red cells inhibited. The antibody which precipitated the Lee virus did not neutralize it, however, nor did it inhibit the chick cell agglutination of the virus. When tested against a rabbit antiserum to normal mouse lung, the PR 8 virus obtained from mouse lung precipitated about fifty per cent of the antibody present. After a single passage of the mouse lung virus in eggs it became indistinguishable by the quantitative precipitin reaction from the fully egg-adapted strain, although some quantitative differences in the chick cell agglutinating activities could be noted. Antibody to normal chorioallantoic fluid was found to precipitate 96 per cent of the virus activity of the egg-grown PR 8 strain. Human antibody to the virus was also shown to precipitate the virus, and the removal of antibody was accompanied by a loss in chick cell agglutination-inhibition power. From this data, Knight formulated a basic concept of virus structure: there is a common denominator to the influenza virus grown in the chicken, the mouse, and man, in that antibodies to any one serological strain neutralize the infectivity and inhibit the cell agglutination reaction. Combined with the virus is also normal tissue protein derived from the particular host species. This combination is firm and results in a structure which is homogeneous by the usual tests. It is not known whether the normal components contributed by the host are functionally essential to the virus. Further chemical and physical data by Knight (64) on PR 8 virus prepared from mouse lungs and from allantoic fluid indicate that the viruses grown in these two sources are of the same size and shape, and have the same infectivity for the chick embryo (although not for mice, since the egg preparations were not mouse adapted). They also have essentially the same chemical composition, although small but definite differences in electrophoretic behavior were found. As noted above, the greatest difference between the viruses prepared from these two sources is in their reactivities to antisera to normal tissues from the host in which they were prepared. The very close correspondences in preparations from such diverse

sources as eggs and mouse lungs was taken as evidence that the substances described were actually the virus units.

Preliminary chemical data on the virus of Newcastle disease in fowls has been reported by Cunha *et al.* (65). It resembles the T_2 bacteriophage in morphology as judged under the electron microscope but differs from it in chemical composition.

A number of studies have appeared on the isolation and characterization of the *B. coli* phage T_2. The active material can be concentrated from culture lysates by sedimentation (66). In the electron microscope characteristic tadpole-shaped particles were observed, with definite morphological differentiation in internal structure. The latter, together with the complexity of the material as revealed by chemical analysis, have led these investigators to conclude that the phage isolated is not a chemical molecule in the usual sense but an organized structure resembling a cell. The sedimentation characteristics have been shown (67) to be quite complex. Chemical analyses (68) have shown the presence of protein, lipid, and nucleic acid of both the ribose and desoxyribose type. The amount of carbohydrate found was in excess of that which could be accounted for by combination with nucleic acid. Other analyses on phage T_2 have been reported by Cohen & Anderson (69) who concluded that all of the nondialyzable phosphorus can be accounted for as desoxyribonucleic acid, which constituted 37 per cent of the preparation. Certain aspects of the problems relating to bacteriophage have been reviewed by Delbruck (70).

Antibodies, Origin and Properties

The reports from the several groups which are actively investigating the site of antibody production have been reviewed recently (2). Bjørneboe, Gormsen & Lindquist (71) have presented further evidence that antibodies may be produced by plasma cells, although they consider the evidence that they may also be produced by lymphocytes, and that the two types of cells may be closely related. Evidence for the increased rate of antibody liberation from lymphocytes after administration of pituitary-adrenal cortical hormones has been given by Chase, White & Dougherty (72). A study of the antigenic properties of human syphilitic antibody globulin has been made by Henriksen (73). Washed Wadsworth-Brown floccules injected into rabbits gave rise to antibodies against the lipoidal antigen and to the syphilitic antibody. The specificity of

the syphilitic antibodies resembled that of certain normal serum globulins. An extensive review of the plasma fractionation work carried out during the war under the direction of Cohn has been given by Edsall (74). Digestion of human gamma-globulin by pepsin has been reported by Bridgman (75) to give molecules of half-size. Most of the antibody function was undiminished [cf. also (76)]. Petermann (77) has found that digestion with papain or bromelin leads to the formation of quarter-size molecules. The preparations were very inhomogeneous. Part of the antibody activity was retained. Data on the hexose and hexosamine content as well as the percentage of tryptophane and phenylalanine have been obtained (78) for a number of immune proteins (cow, horse, and human). The values are close together but are believed to be distinguishable for each species. The characteristics of the ultraviolet light absorption can be accounted for on the basis of the contents of phenylalanine, tyrosine and tryptophane (79).

Morgan & Shütze (80) have reported that anti-Shiga or antityphoid sera contain antibodies which do not agglutinate the homologous organism, or do so to low titer, and that much higher titers can be detected by the further agglutination of the "sensitized" organisms with an antiserum to human gamma globulin. Some similarities with "soluble univalent" antibodies were pointed out. Although as discussed below there is little doubt as to the existence of these incomplete antibodies in various sera, the type of evidence presented in this report is not sufficiently conclusive to permit assigning a definite explanation to the results. It may be doubted, for example, that an increase in titer can be taken as evidence of an additional antibody, since it is well known that the sensitivities to agglutination of different organisms or particles vary, and there is no reason why organisms partially coated with one human antibody should not exhibit an independent (in this case, greater) titer to a second antiserum to a human globulin. The existence of incomplete antibodies which combine with an antigen without giving the usual direct visible evidences of reaction has been established for some time (50, 80, 81). They have assumed added importance in discussions on their relation to Rh "blocking antibodies" (2). The problem of why horse antibodies to various proteins may or may not exhibit a prozone has been examined by Treffers, Heidelberger, & Freund (82). It was found that the subcutaneous injection of rabbit serum albumin into horses produced

antibody exhibiting a typical prozone, of the type given by diphtheria antitoxin. The subcutaneous injection of this antigen did not give rise to any antibody detectable by the usual methods. The intravenous injection of rabbit serum globulin into other horses did result in antibody resembling anticarbohydrate antibodies in that no prozone was evident. The subcutaneous injection of the antigen gave rise only to incomplete antibody which could be detected by its addition to flocculating antibody of the usual type. Chemical and physical differences could also be demonstrated for the various antibodies. Although the route of injection is apparently not the only factor responsible for the character of the antibody produced, it appears to exert a determining influence. The data on the interesting effects produced by heat on antisera have been reviewed and extended by Follensby & Hooker (83). Some of the heated sera resemble incomplete antibodies in their reactivities with antigens. Similar modifications in reactivity by irradiation of sera have been reported by Tyler (84). Boyd (85) has been unable to modify the properties of isohemagglutinating antibodies by this procedure.

The extensive recent developments in our knowledge of complement have been reviewed (2, 86, 87). Spectrophotometric methods for the standardization of complement in terms of the fifty per cent hemolytic unit have been developed (88, 89). In a most suggestive study, Mayer *et al.* (90) have shown that divalent ions, especially calcium and magnesium, are essential for complement hemolytic activity, and that the ionic levels are not optimum under many usual experimental conditions.

Immunity to Anthrax

The important problem of active immunization against anthrax has engaged the attention of numerous investigators since Pasteur's classic researches (94) in which he showed that immunizing with living avirulent bacilli would protect animals against subsequent inoculation with living virulent organisms. Killed anthrax organisms generally have no immunizing effect, and although a few claims of successful experiments have appeared from time to time the results were irregular and not uniformly reproducible. A step forward was taken in 1904 by Bail (95) who showed that edema fluid from anthrax lesions would confer active protection in animals. This work has been abundantly confirmed [for literature see (96, 97)]. Later Bail & Weil (98) reported the presence in ex-

tracts of anthrax lesions of substances, which they termed aggressins, which interfere with natural host resistance. This work, however, gave rise to considerable controversy and it is only recently that the fundamental factors underlying these observations have been elucidated. The results are of such interest from many points of view that it is worth considering them in some detail.

Preis (99) first pointed out the relationship between capsule formation of *B. anthracis* and the production of edema in living tissue. Capsular material extracted from the organism was capable of neutralizing bactericidal substances obtained from the host. It was later shown by Tomcsik & Szongott (100) that the capsular material from *B. anthracis* contained 10 per cent N and failed to give a positive biuret or Molisch test. It was found by Ivanovics *et al.* (101, 102) to be a polypeptide containing 40–50 *d*(−) glutamic acid units. Similar materials were also obtained from the related but avirulent organisms *B. mesentericus* and *B. subtilis*. Kabat (50) has shown this glutamyl polypeptide to be homogeneous by electrophoresis. An additional, somatic, polysaccharide has been isolated from edema fluid in anthrax infections by Grabar & Staub (103, 104, 105). Both it, and the glutamyl polypeptide occur free and in combination with protein. Both antigens were found to be precipitated by the euglobulin fraction of antianthrax sera, although the protective power of such sera is concentrated in the pseudoglobulin fraction. It appeared, therefore, that they were not concerned with protection; in a later paper, however, Grabar & Staub (106) have reported successful immunization of animals with edema fluid containing the somatic polysaccharide and postulated that the active antigen consists of a combination of the polysaccharide with protein.

Before considering further the possible role of these substances in protection against anthrax infection, attention must first be directed toward the more biological phases. They have been considerably clarified in a series of papers reported by a group working at Camp Detrick, Md. (97, 107–111). It has been appreciated for many years that some species of animals such as mice, guinea pigs, rabbits and sheep are relatively susceptible to anthrax, the disease taking an acute course with death ensuing within forty-eight to ninety-six hours after experimental inoculation. Dogs and swine are more resistant, the disease in these animals taking a chronic course often ending in recovery. Various theories and experiments

have been offered to account for these differences. In their first report, Cromartie, Bloom, & Watson (107) presented histopathological data that in both resistant and susceptible animals species the initial stage of infection consists in the germination of the spores introduced, with multiplication of the resulting organisms. The latter are encapsulated and stain readily in tissue sections.

In the susceptible animal this process continues until the death of the animal, although the mechanism of death from this infection still remains obscure. In a physiological study of susceptible animals this group (111) presented evidence that the time of death is not correlated with the number of organisms developing. In fact, in a few animals death ensued after a relatively small number of organisms had developed and it is evident that a theory of mere mechanical obstruction is not tenable. The most suggestive findings were disturbances in carbohydrate metabolism as indicated by a hyperglycemia, and in calcium metabolism as indicated by lassitude, drowsiness, excessive response to stimuli, and a tendency of the animals to bleed. In agreement with the latter, it could be shown that some modification of the course of the disease could be effected by injections of calcium salts. Further investigation of these factors is obviously indicated.

In animals which are relatively resistant to anthrax, proliferation of the organism also continues for two to four hours. After this time, however, the bacilli lose the capsules, exhibit the staining characteristics of dead and disintegrated organisms, and gradually disappear from the lesions. Although the area around the organisms becomes infiltrated by leucocytes, phagocytosis in this, unlike many other infections, is not a distinguishing feature. It is the conclusions of this group that the species difference in resistance to anthrax is in some way associated with the ability of the host to affect the capsules of the organism. The character of the tissue changes in the susceptible animals suggests a diffusible substance liberated by the organism as the inciting agent. In later studies a more detailed examination was made of these two agencies, one a host reaction affecting the organism, the other, an agent elaborated by the organism which produces edema in the host.

In a further study of the Camp Detrick group (108) evidence was presented that a filterable substance could be extracted from anthrax skin lesions in rabbits which would produce on injection into the skin of normal rabbits tissue damage which very closely

resembled that produced by an active infection with anthrax organisms, except that the bacillary bodies themselves were absent in the sections. This crude extract was actively antigenic in rabbits, a course of three weekly injections totaling three ml. resulted in protection of ten out of ten animals against ten to one hundred lethal doses of anthrax spores. Protection of a very small series of immunized animals (one per dose) against respiratory challenge doses as high as twenty lethal doses could also be demonstrated. In addition to the greater survival rate, animals which had been immunized with this crude extract gave other indications of immunity in that the lesions at the site of intradermal challenge injections showed extensive destruction of the anthrax bacilli, a picture similar to that observed in naturally resistant species. Tissue damage was minimal. In so far as one can reason from identical pathological pictures that two inciting agents are identical (which need not necessarily be the case since the tissue may be limited in its types of response) the data indicate that the defensive mechanisms of naturally resistant and of immunized animals are identical. The basic defensive mechanism is thought to be a reduction in the offensive properties of *B. anthracis*—specifically an inhibition of capsule formation—which then permits the other factors in natural resistance of the host to operate and destroy the organism.

In a third study (109) a separation was made of the two factors in edema fluid which have the properties of causing tissue damage, or of inducing protection in animals. Electrophoretic examination at pH 8.1 of crude extracts of anthrax lesions yielded patterns similar to those found for normal rabbit sera, except for a component migrating more rapidly than albumin, with a mobility of 16.4. The electrophoretic area was about 8 per cent of the total nondialyzable components. When the crude extract was precipitated with calcium in the presence of phosphate both tissue damage and protective activities were found in the precipitate; the supernatent, however, contained only the protective activity free of tissue damage activity. Two distinctive substances were therefore indicated. In order to identify the protective activity with one of the electrophoretic components (the fast moving component had been removed by the calcium phosphate precipitation and could therefore not be associated with this property) a separation was effected in a macro Tiselius cell. A fraction containing all

protein of alpha-three mobility or faster had no protective activity. However fractions containing largely beta globulin with a small amount of gamma globulin, or mostly gamma globulin with a small amount of beta globulin protected animals to the same degree. It was therefore concluded that the protective antigen migrated between the beta and gamma globulins although no such separate component could be distinguished in the electrophoretic patterns. The protective antigen is destroyed by heating at 57°C. for thirty minutes, and by trypsin. It is stable to formalin, however, and can be lyophilized. With proper control of temperature it will also withstand precipitation with alcohol, which offers promise for the fractionation studies.

The immunization of animals with the crude edema fluid referred to above was attended with loss of weight and death in a certain percentage of the animals. This toxicity disappeared after the removal of the tissue damaging material with calcium phosphate and animals could be immunized with the protective factor without difficulty. A small series of animals (seven to fifteen of each species) was immunized with the protective factor obtained from anthrax lesions in rabbits. The data indicate that although normally highly susceptible, the guinea pig can be immunized more effectively than the mouse or hamster, although not as effectively as rabbits. Even a single injection of protective antigen into sheep apears to confer appreciable protection.

As has been appreciated by other workers, following the course of a protective antigen during fractionation trials by means of protective antigenicity tests is an inconvenient procedure in that the biological readings are not completed until days or weeks after the chemical fractionations they are supposed to aid. Again, even in favorable cases, quantitative protection data which can be compared with series run under other conditions are difficult to obtain. Attempts were therefore made to identify the protective antigen by means of some ***in vitro*** serological reaction. Three sera were available. Serum A was secured by injecting rabbits intravenously with crude extract. After bleeding, the animals were challenged with 10,000 lethal doses of spores. All survived. Serum B was secured by injection with killed anthrax bacilli. No immunity even to small doses could be established in these rabbits after removal of the test serum. The rabbits which furnished serum A and survived after the large challenge dose were again

injected with crude extract containing the protective antigen, for two courses lasting seven to twelve weeks, followed by challenge doses of up to 10^9 lethal doses of spores. All animals survived. Two weeks after the last dose the animals were bled to furnish serum pool C. The electrophoretic pattern at this time gave no evidence of any change in globulin concentration attributable to antibody. Precipitin tests with crude antigen were entirely negative with serum A obtained from highly immune animals. Precipitation tests were positive, however, with serum B, obtained from animals showing no immunity. Similar differences were obtained by complement fixation, except that serum A gave a reaction at low titer. Serum C injected into guinea pigs resulted in passive protection in fifty to eighty per cent of the animals against six lethal doses of spores. Attempts to influence this passive protection by first absorbing the serum with crude extract were unsuccessful [cf. also (96, 103, 104, 105)] as were attempts to reduce the antibody N precipitated by the crude extract after heat inactivation of the protective antigen. It appears to the reviewer that the latter point is not entirely convincing since the protective antigenicity of this material might be destroyed before its (hypothetical) antibody binding power.

As has been mentioned earlier, immunity to anthrax can be produced by injection with living organisms (which initially at least must be avirulent if the animal is to survive) or with extracts of anthrax lesions containing the protective antigen. Killed organisms are without effect. This situation is not exactly unique, having been observed in attempts at immunization against many virus infections and some bacterial diseases such as tuberculosis. In the case of viruses it is becoming clear, however, that the failure to immunize with killed material was due, at least in some instances, to the minute amounts which were administered in the crude preparations available. When living avirulent vaccines are administered they multiply in the host, resulting in an effective increase in the antigenic dose. The successful use, however, of modern concentrated preparations of killed vaccines to such agents as influenza virus has demonstrated the correctness of the theory that the difference between killed and living material can be a quantitative rather than a qualitative one. There may, of course, be instances in which the injected living vaccine localizes in a site different from that taken by a killed vaccine, and this may influence

subsequent response of the host. In the instance of tuberculosis, however, the factors responsible for the difference in antigenic effectiveness between living and dead organisms are not at all clear and constitute a most important problem for continued investigation.

The production *in vitro* of anthrax cultures which possess the protective antigen would constitute a significant advance in our understanding of the relationship between host and parasite. Experiments to this end have been conducted by a British team working independently of but in coordination with the group at Camp Detrick. Gladstone (96) has recently reported the production in anthrax culture media of protective factors similar to that present in the edema fluid described above. Two nutrient factors were found necessary for its production; one, a nondialyzable component, was present in sheep, rabbit or human sera or plasma and is apparently associated with the serum proteins; the other was removed on dialysis but could be replaced with sodium bicarbonate. The size of the culture inoculum, the age and pH (>6.5) of the culture were found to be important factors. No virulent strain failed to produce the antigen but there was no other relationship between production of antigen and virulence, capsulation or sporulation. Avirulent strains which in the living state were effective immunizing antigens also produced the protective factor in serum cultures. Although the protective factor is present in the culture fluid, no protective activity could be demonstrated in the killed washed organisms. Further attention is being given to the possibility that the antigen is destroyed by a proteinase known to be produced by *B. anthracis*. Protective tests in animals indicated that rabbits receiving three weekly doses of one ml. of culture filtrate would survive as high as 100,000 lethal challenge doses. Nevertheless the serum from such animals was completely devoid of any antibody which would confer passive protection in other animals. Attempts to demonstrate protective antibody in ground spleen or liver were negative. A further course of immunization with living organisms resulted, however, in antibody which conferred passive protection in other rabbits. In agreement with the results of the American and French investigators attempts to remove or neutralize the antibody from sera conferring passive protection were all negative.

Some indication of a possible host defense against anthrax has

been obtained by the Camp Detrick group (110). As reported above they had noted that although the organisms were not phagocytosed they were usually accompanied in the lesions by large numbers of leucocytes, and that in resistant animals the organisms exhibited degenerative changes. It appeared possible, therefore, that the leucocytes might possess some factor which injured the organisms. Similar ideas have been advanced previously by other workers but only the more recent data bearing on the point can be considered here. It was found (110) that actively anthracidal fractions could be extracted from leucocytes as well as from a number of tissues such as pancreas and thymus derived from various species, including the dog, hog, and calf. Even the crude material had an almost immediate effect on the oxygen uptake of respiring anthrax organisms, and plate counts demonstrated a rapid reduction in viable organisms. From the method of preparation it appeared possible that the active fraction could be either lysozyme or ribonuclease but known preparations of these two enzymes failed to exhibit anthracidal activity. By ethanol fractionation some purification of the active material could be effected. The electrophoretic pattern was somewhat diffuse and on prolonged electrophoresis evidence of three peaks with close mobilities was obtained. The isoelectric point was estimated to be 11.2. The best preparation contained 15.3 per cent nitrogen and 0.94 per cent phosphorus. Amino acid analysis gave arginine, 3.5 per cent, histidine, 1.04 per cent, and lysine, 29.3 per cent. It was concluded that the material is a basic polypeptide with a high content of the latter amino acid. Preliminary studies have indicated that it may have therapeutic possibilities since forty-eight per cent survival of mice was obtained after challenge with one hundred lethal doses.

In concluding this somewhat extended survey of recent developments in the immunochemistry of anthrax, mention must be made of a study of the material related to the anthrax capsular polypeptide which has appeared (97). It will be recalled that the Camp Detrick group had separated the crude edema fluid from anthrax lesions into two fractions, one containing the protective factor, the other a substance which was responsible for the edema and other toxic manifestations. The latter material was found to have an electrophoretic mobility greater than that of serum albumin and could be separated from the protective factor by precipi-

tation with calcium phosphate. Various considerations have led to the belief that the inflammatory material is related to the capsules of the organism (99). In this study (97) it was found possible to further purify the inflammatory factor and identify it as a glutamyl polypeptide. Analyses by the method of isotope dilution indicated 81.6 per cent of *d*(−) glutamic acid, although the total glutamic acid content as determined enzymatically was appreciably lower, 69 per cent. Similar analyses on the glutamyl polypeptide isolated from the capsule of *B. subtilis* grown on Sauton's medium yielded 102 per cent total glutamic acid, of which 85 per cent was *d*(−) glutamic acid, and (by difference) 15 per cent was *l*(+) glutamic acid. A detailed comparison was made of other chemical and physical properties of these two polypeptides and it was concluded that they were very close but not identical in behavior. Small but significant differences were also found in comparing the properties of these two materials with that isolated by Ivanovics & Bruckner (102), which was composed entirely of *d*(−) glutamic acid units. The latter investigators had provided evidence that the glutamyl polypeptides derived from strains of *B. anthracis*, *B. subtilis*, and *B. mesentericus* grown on simple synthetic media were identical in all respects. The most striking differences between the glutamyl polypeptide from *B. anthracis* and from *B. subtilis* were found (97) in their biological properties. The material from the anthrax organism produces inflammatory changes in the skin and interferes with blood clotting (97). Similar amounts of the *B. subtilis* polypeptide had neither of these properties, nor in contrast to the anthrax polypeptide had they any ability to neutralize the bactericidal effect of leucocytes. In evaluating the significance of this latter property, Watson *et al.* (97) feel that it is a factor of some possible importance in determining the fate of the host in anthrax infections.

Two of the many points brought out in these papers may deserve a further word of comment. The first is the clear illustration in these studies of another instance in which protection, either actively induced or passively transferred, may not be correlated with any demonstrable serological test. In fact, in this case the antibody could not be absorbed out by any available antigenic fraction. Other workers in the field of immunology have been so used to estimating protection in terms of such tests that they sometimes find it difficult to realize the limitations which may be

involved. The situation is frequently made more difficult and deceptive by the fact that an *in vitro* serological test is obtained, although closer analysis shows that the test bears little or no relation to the protective activity of the serum. Antibodies of this type have been found in a number of studies, including those of Kabat on meningococcus (112), Smadel and Shedlovsky on vaccinia (113) and Treffers on chemically modified dysentery antigens (36). The second point, to which reference was also made in the discussion on bacterial nutrition above, is that bacteria such as the anthrax organism growing in a susceptible host may produce substances of great biological importance which cannot be demonstrated in cultures made in the usual media, nevertheless as Gladstone's study (96) indicates, modifications can be made which will permit the production of at least one of the factors—in this instance, the protective antigen. Although considerable progress in the study of the mechanism of immunity and infection in anthrax is evident, the complete picture is by no means clear. Of major interest remain the mechanism of the immunity without apparent antibody production elicited by injection of the protective factor, and the reasons why further immunization with living organisms produces transferable antibody.

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SOME ASPECTS OF ACTIVE IMMUNIZATION

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Active immunization against diphtheria, scarlet fever, tetanus, pertussis, typhoid fever, smallpox and rabies was admirably discussed in 1946 in Edsall's (1) review covering 444 papers. Other aspects of active immunization are dealt with elsewhere in this publication. This chapter is therefore devoted to the function of cells in antibody formation, to immunization with the aid of adjuvants and to the inhibition of sensitization by feeding the antigen.

Cells Responsible for Antibody Formation

Phagocytic tissue cells.—Until recently it was generally held that antibody formation is the function of the "reticuloendothelial system" i.e., phagocytic tissue cells. The view was based on histological evidence of phagocytosis and diminished antibody formation when the phagocytic cells are burdened with colloidal matter before making the antigen available to them. These points are not direct evidence and the blockading of cells with colloidal matter may have effects not related to the phagocytic cells. Beard & Rous (2) approached the problem more directly by testing the capacity of isolated Kupffer cells to produce antibodies in tissue culture. The negative results obtained according to the authors are not decisive and may be due to the conditions peculiar to the tissue culture method. For example, the cultured Kupffer cells were devoid of their capacity to phagocytose.

Spleen and lymphocytes.—Rich (3) called attention to the possible role of lymphocytes in antibody formation. It was later noted (4) that intravenous injections of sterile foreign proteins cause "acute splenic tumors" in rabbits and that splenic enlargement and antibody titers both increase with repetition of the injections. Rich *et al.* (5) concluded on the basis of motion picture studies of living cells that the new spleen cells are nonphagocytic lymphoid cells and suggested that lymphocytes are probably concerned with antibody production. Others (6, 7) believe that cells

found in "acute splenic tumor" are mainly mononuclear phagocytes and lymphoblasts.

Topley (79) found that the spleen alters antigens. Spleens were removed from rabbits after intravenous injections of killed bacteria. Such splenic suspensions injected into normal rabbits led to accelerated antibody formation of short duration. No secondary response was found on reimmunization.

Lymph node.—McMaster *et al.* (8, 9) obtained direct evidence for the formation of antibodies in the lymph node. They found that shortly after the injection of bacterial antigens into the ears of mice the concentration of antibodies is higher in the draining lymph nodes than in the blood. Different species of bacteria were injected into each ear of mice, thus excluding the possibility of accumulation of antibodies from the blood by the draining lymph nodes. Burnet *et al.* (10, 11) using viral antigens confirmed these conclusions. The kind of cells producing antibodies in the lymph nodes was not determined.

Inflammatory cells of the antigen-depot.—Lymph nodes may be especially adapted for antibody formation because they accumulate and store particulate matter. The cells in lymph nodes engaged in the formation of antibodies may be of the same kind which meet antigens and possibly manufacture antibodies in other organs.

Killed *M. tuberculosis* suspended in paraffin oil injected intracutaneously produce in rabbits local lesions. There is here a good opportunity to demonstrate antibody formation in the skin lesion because antibodies appear in the blood only two to three weeks after the injection of the antigen. In these skin lesions complement-fixing antibodies in high titers were found before they became demonstrable in the blood (75). Lesions produced in the same rabbits by intracutaneous injections of various irritants contained no antibodies or antibodies in low titers. Thus again the accumulation of antibodies from the blood by the lesion caused by the antigen can be excluded. The lesions resemble tubercles and are rich in large phagocytic mononuclear cells.

Lymphocytes.—Ehrich & Harris (12) confirmed and extended the studies by McMaster *et al.* Sheep erythrocytes, killed *Shigella paradysenteriae* and *S. typhi* were used as antigens. The antigens were injected into the pad of the rabbit's foot, and homologous antibodies were found a few days thereafter in the extract of the

popliteal lymph node and in lymph collected from the efferent, but not the afferent, lymphatic vessel of that node. These titers at times exceeded that found in the serum. At the same time, marked activity of the lymph node was indicated by considerable increase in size, with a profusion of new secondary follicles, and a sharp increase in the number of cells in the efferent lymph. According to the authors, 99 per cent of the cells in the efferent lymph were lymphocytes.

On collecting the lymph cells by centrifugalization it was found that a higher concentration of antibody existed in these cells than in the lymph plasma surrounding them. The difference in titers was greatest at the time of greatest rate of formation of antibodies. The authors concluded that the lymphocytes were the primary site of the antibodies found (13).

To test the possibility of antibody formation by the macrophage, vaccines in water/oil emulsions were injected into the foot pad. Despite the marked attraction of macrophages to the site of injection by the mineral oil, antibodies were not found in this locality following the injection of emulsified vaccines (14, 15).

Dougherty & White (16 to 21) reported a number of observations on the possible rôle of lymphocytes in antibody formation. Injection of adrenal cortical extract or pituitary adrenotrophic hormone causes, within a few hours, damage ("dissolution") to lymphocytes in the lymphatic organs, a leukopenia, and an increase in the serum proteins and in antibody titers. Furthermore these authors reported that they had extracted a material giving an electrophoretic pattern corresponding to gamma globulin from suspensions of cells consisting mainly of lymphocytes. In one series of experiments an "anamnestic" reaction was produced, i.e., within a few hours after the injection of the hormones, antibodies appeared in the blood of animals which had been immunized some time before, but whose blood had been free of demonstrable antibodies at the time of injection of hormones. The question arises whether the cortical hormone has effects other than causing the "dissolution" of lymphocytes, particularly because the "anamnestic" reactions can be produced with a great variety of agents. Exact information concerning the types of cells extracted seems to be essential.

Polson (25) recently found that in new born calves and horses the blood plasma is lacking in globulin. In general it is said that

germinal centers in the lymphatic organs are not found in new born animals. The correlation of changes in serum globulin concentration, the morphology of lymphatic organs and the capacity for immunological response would be an interesting subject for study.

Plasma cells.—Bjørneboe *et al.* (22) using a different approach, studied antibody formation in relation to plasma cells. Killed pneumococci were injected into rabbits every other day for from one to eight months. The rabbits were exsanguinated and the fat of the renal pelves, the retroperitoneal fat, and the mesenteric lymph nodes, thymus, lungs, liver, bone marrow and kidneys were studied histologically and extracted. The agglutinin contents of the extracts and of the sera were determined by the quantitative method of Heidelberger & Kabat. Large numbers of plasma cells were found in the fat of the renal pelvis only. However, approximately 10 per cent of the cells in this tissue were lymphocytes. It was found that the antibody contents of the organs except the renal pelvic fat ranged from 5 to 14 per cent; the antibody content of the pelvic fat, rich in plasma cells, had from 37 to 62 per cent of that found in the sera. According to the authors there were no plasma cells in the thymus, and the antibody content of this organ, so rich in lymphocytes, was low. The authors advanced the hypothesis that antibodies are produced by plasma cells.

Since the plasma cells are not known to be phagocytic it is necessary to assume (as in the case of the lymphocyte theory of antibody formation) the operation of a process to make particulate antigens soluble in order to prepare them for the cells. Furthermore, it would seem less complicated to study antibody formation after one or only a few injections of antigens, and to extract tissues in the early part of immunization before the antibody content of the blood exceeds that of the extracted tissues.

Sensitizing antibodies and inflammatory cells.—Another type of experiment which indicates the rôle of inflammatory cells in the antibody formation of hypersensitivity is the transfer, by means of cells, of sensitivity to tuberculin and to simple chemical compounds. Chase (23) succeeded in transferring tuberculin sensitivity from actively sensitized to normal guinea pigs by intraperitoneal or intravenous injections of cells obtained from the peritoneal cavities of the sensitized pigs. The cells were comprised of 15 to

30 per cent of polymorphonuclear leucocytes, 20 to 35 per cent of lymphocytes and 50 to 65 per cent of large mononuclear cells. Since such cells did not transfer sensitization after gentle heating or freezing it is possible that they function not only as the bearers of sensitizing antibodies, but also as the elaborators. Landsteiner & Chase (24) had previously succeeded in transferring cutaneous sensitivity to picryl chloride by using cells from the peritoneal exudates of guinea pigs sensitized with picryl chloride plus tubercle bacilli. The type of cell responsible for the transfer has not been determined in either experiment.

Although the rôle of cells in forming antibodies in tissue cultures has not been clearly determined, it is significant that chicken fibroblasts elaborate serum proteins in such cultures: Therefore the production of serum globulin is not likely to be the sole function of lymphocytes or plasma cells [Landsteiner & Parker (26)].

It would seem likely that a direct demonstration of the type of cells involved in antibody formation might be possible if the interaction of antibody bearing cells and antigen could be made visible, perhaps with the aid of antigen coupled with dyes.

Immunization with the Aid of Adjuvants

Various adjuvants.—Interest in promoting antibody formation by addition of unrelated substances to antigens has never been lacking. Gonzalez *et al.* (28) and Armangué *et al.* (29), showed that the Forssman hapten adsorbed on kaolin and charcoal is antigenic. Jacobs (27) found that the slight antigenic activity of heteroalbumose is increased by adsorption to these agents: tapioca, salts of calcium and magnesium, tannin, killed *S. typhi*, and lanolin. Tapioca increases diphtheria antitoxin formation according to Ramon *et al.* (30). The adsorption of toxoids on alum phosphate is very effective in increasing immunologic responses [Glenny *et al.* (31)]. Staphylococcal toxin added to poor antigens has an enhancing effect on immunization (31a, 31b).

Paraffin oil.—Some years ago Coulaud (32) discovered that sensitization to tuberculin in guinea pig develops to an unusually high degree when killed tubercle bacilli incorporated in paraffin of high melting point are deposited into the subcutaneous tissue. Later Saenz (33) and others (34, 35, 36) using paraffin oil extended this observation. It was also found that paraffin oil enhances and

remarkably prolongs the formation of complement fixing antibodies (34). It was reported by Ramon *et al.* (30) that paraffin oil has some but not a striking effect on antitoxin formation.

More than thirty years ago, typhoid vaccines mixed with vegetable oils to reduce their toxicity, were tested in man and lower animals. These "lipo-vaccines" were no more antigenic nor less toxic than those routinely used.

Living tubercle bacilli.—Lewis *et al.* (37, 38, 39) discovered that living, virulent tubercle bacilli injected into the peritoneal cavities of guinea pigs brought about an enhancement of antibody formation to various antigens subsequently introduced by the same route. Killed tubercle bacilli were almost ineffective. Dienes (40, 41) confirmed and extended these observations. He found that sensitization to the antigens contained in horse serum and egg white is both of the anaphylactic and of the tuberculin type when the antigens are injected into lesions (in the subcutaneous tissue) produced by the injection of a large number of tubercle bacilli. Just as in the experiments of Lewis *et al.*, the potentiating effect failed to appear or was equivocal when killed tubercle bacilli were substituted for living ones.

Killed Mycobacteria in Water/Oil Emulsion as an Adjuvant

It seemed desirable to substitute killed mycobacteria for living tubercle bacilli because the multiplication of tubercle bacilli and the tuberculous state itself complicate experiments; in addition it is inconvenient to work with tuberculous animals. Since paraffin oil (liquid petrolatum) added to mycobacteria enhances the tissue reaction (Grassberger (42)), the sensitization to tuberculin and antibody formation against tubercle bacilli (34, 42), it was expected that horse serum injected into a lesion (in the subcutaneous tissue) produced by deposition of paraffin oil containing killed tubercle bacilli would result in increased antibody formation and altered sensitivity to the serum. Such experiments failed in our hands (43) although Landsteiner & Chase (24) obtained enhanced sensitization to simple compounds when similar experiments were carried out using the peritoneal cavity as the site of the two injections. The effect of living tubercle bacilli on antibody formation and sensitization can be obtained with killed tubercle bacilli or saprophytic mycobacteria by the water/oil emulsion method. In

such preparations most of the mycobacteria are in the continuous oil phase. Not only is the full effect of mycobacteria manifested in water/oil emulsion but it is of amazingly long duration.

The technique of preparing water/oil emulsion consists of mixing an aqueous solution or suspension of the antigen with paraffin oil which may contain killed mycrobacteria, using an emulsifying agent such as Aquaphor,[1] Falba[2] or anhydrous lanolin. The proportions are important: if Falba is used the proportions should be aqueous phase 2 to 3.5:Falba 1:paraffin oil 2 or less (44, 45,

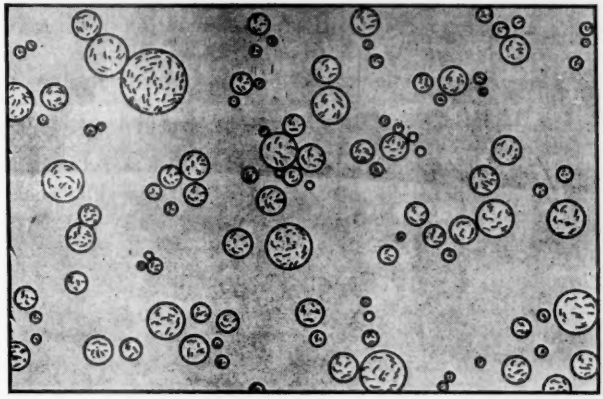

FIG. 1. EMULSION OF WATER IN OIL: TYPHOID BACILLI IN DROPLETS OF SALT SOLUTION IN A MENSTRUUM OF FALBA AND PARAFFIN OIL
The typhoid bacilli are in Brownian movement

46). It was found that when four parts of paraffin oil are used rather than two, antibody formation is retarded and slightly diminished. The blending can be accomplished in a mortar, Waring blendor or in a syringe by repeated withdrawals and ejections (47). The water-in-oil emulsion containing antigen (*S. typhi*) is depicted in Fig. 1. When peanut oil is substituted for paraffin oil the enhancing effect is lost even in the presence of beeswax so widely used for the repository injection of penicillin (48).

[1] Aquaphor is an ointment base composed of derivatives of wool fat mixed with hydrocarbons (Duke Laboratories).

[2] Falba is an adsorption base derived from lanolin, marketed by Pfaltz and Bauer, Inc., N.Y., N.Y.

The mycobacteria studied include human and bovine types of tubercle bacilli, *M. phlei* and *M. butyricum*. Friedewald (49, 50) compared the human tubercle bacillus and *M. butyricum* and found the latter more effective in promoting antibody formation against influenza virus. The three kinds of mycobacteria were compared regarding antibody formation against *S. typhi; M. butyricum* appeared to be slightly more effective than *M. tuberculosis* and *M. phlei* much inferior (48). Halbert, Mudd & Smolens (51) working with *Shigella* antigen did not find tubercle bacilli more effective. When horse serum is injected into guinea pigs the administration in water/oil emulsion increases precipitin formation but has little effect on sensitization; the addition of killed tubercle or timothy bacilli however conspicuously alters sensitization to horse serum (52).

Sensitization.—Very striking is the effect of mycobacteria on sensitization to some simple compounds such as picryl chloride and 2:4 dinitrochlorobenzene and conjugates of these substances with protein. When these are injected intraperitoneally a state of anaphylaxis alone ensues, while the use of mycobacteria produces in addition sensitization of the contact dermatitis type [Landsteiner & Chase (53)]. The combination of killed tubercle bacilli, Aquaphor and paraffin oil injected subcutaneously produces contact type of sensitization to Aquaphor [Chase (54)]. The results were similar in regard to lanolin when guinea pig brain, lanolin and killed *M. butyricum* in water/oil emulsion were injected subcutaneously into guinea pigs (55).

Iso-allergic encephalitis.—Employing water/oil emulsions containing killed mycobacteria, a method became available for the rapid production of iso-allergic encephalomyelitis. Morgan (56) injected rhesus monkeys with homologous brain and cord in water /oil emulsion containing killed tubercle bacilli and found that these animals developed neurologic symptoms (blindness, ataxia, nystagmus, paralysis of the limbs). Lesions in the brain and cord included thrombosis, perivascular cuffing and demyelination. Kabat *et al.* (57) independently from Morgan, made similar observations. The Kopeloffs and others (55, 58) produced iso-allergic encephalitis in guinea pigs, but in this species the symptoms are less spectacular. Guinea pigs become hypersensitive to the intracutaneous injection of rabbit brain when this is used to induce encephalitis. The reaction is of the delayed necrotic type, as seen in guinea pigs

sensitized to horse serum by the combination of horse serum and tubercle bacilli in water/oil emulsion. For the rapid production of allergic encephalitis in both the monkey and the guinea pig the presence of mycobacteria in the sensitizing injection seems to be essential, although by the use of numerous injections (more than thirty), Schwentker & Rivers (59, 60) produced this phenomenon without adjuvants. This iso-allergic sensitization is in keeping with the fact that brain is known to contain organ specific antigens. In monkeys and in guinea pigs serum from actively sensitized animals injected into normal ones does not produce paralytic symptoms. It is possible that this type of sensitization, like that to tuberculin and to simple chemical compounds (contact type of sensitization), can be transferred by means of cells [Landsteiner & Chase (24), Chase (23)]. The fact that acid fast bacilli function as adjuvants in the sensitization produced with simple compounds, that they cause tuberculin sensitivity, which can not be induced with tuberculo-protein alone, and that they have a part as well in the production of iso-allergic encephalitis points to this possibility.

Antibody formation.—The water/oil emulsion technique was originally devised to blend antigens in aqueous phase with oil containing tubercle bacilli. It was soon found that for sensitization the tubercle bacilli are essential but for increasing and prolonging antibody formation water/oil emulsions without mycobacteria are very effective. The following studies deal with antibody formation employing antigens in water/oil emulsion with or without mycobacteria.

Groups of rabbits were given two simultaneous, subcutaneous injections of killed *S. typhi* (*a*) in salt solution (*b*) in water/oil emulsion or (*c*) in water/oil emulsion containing killed *M. tuberculosis* (45). The results are shown in Fig. 2. In the first group the agglutinin titers reached a peak in two weeks and then began to fall; the preimmunization level was reached in about six months. In the second group the titers continued to rise for about six to eight weeks and remained at peak levels for several months; agglutinin formation was still going on three years after the injection of antigen. The titers in this second group were at least ten times higher than in the group with the antigen in saline. Antibody formation in the third group which received the antigen in water/oil emulsion but containing, as well, killed *M. tuberculosis* was similar to that in the second group but the titers were slightly higher.

Thus there is no doubt as to the effectiveness of the water/oil emulsion in promoting antibody formation. For an exact evaluation of the results, agglutinin nitrogen determinations rather than

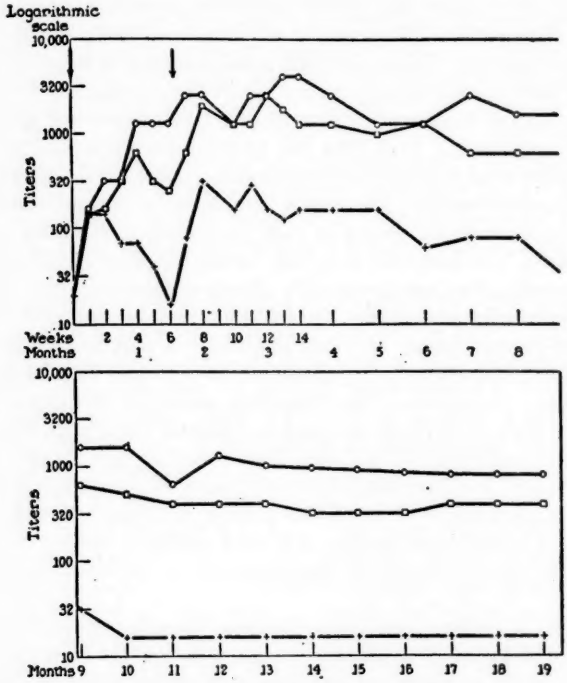

FIG. 2.—AGGLUTININ TITERS (MEAN) IN RABBITS AFTER SUBCUTANEOUS INJECTIONS OF FORMALIN KILLED TYPHOID BACILLI

A +—+ suspended in salt solution
B □—□ combined with Aquaphor and liquid petrolatum
C ○—○ combined with Aquaphor and liquid petrolatum containing killed tubercle bacilli
↓ Injection

titrations are needed. Similar experiments using a soluble antigen, diphtheria toxoid (45), showed the effectiveness of water/oil emulsions; there was however little if any increase in the potentiating effect produced by the addition of killed tubercle bacilli to

the emulsion. Antitoxin formation was better sustained following the injection of diphtheria toxoid in water/oil emulsion than after the injection of alum precipitated toxoid.

It is an interesting observation that when rabbits are injected subcutaneously with diphtheria toxoid in water/oil emulsion, a second injection of the same material given one month later increases the antitoxin titers. When however the same adjuvants are used but the antigen is killed typhoid bacilli, the second injection produces little or no rise in the agglutinin titer unless the two injections are spaced widely apart (more than six months). If the first subcutaneous injection of killed typhoid bacilli or *H. influenzae* type B in water/oil emulsion is however followed a month later by repeated *intravenous* injections of killed *S. typhi* or *H. influenza* B in salt solution, very high agglutinin titers are produced (61). Water/oil emulsions containing killed tubercle bacilli were effective with respect to antibody formation against several "poor" antigens such as ovalbumin (62), alcoholic extract of brain (63) and histaminazo-protein (64). Landsteiner & Chase (24) obtained highly potent sera bearing the anaphylactic type of sensitizing antibodies against certain allergenic chemicals as citraconic anhydride and Chase (65) against ragweed pollen and tuberculin by this method.

Immunization against viruses.—Adjuvants have been applied to immunization against influenza and Japanese B encephalitis virus. Friedewald (49, 50) found that mice immunized with influenza vaccine in water/oil emulsion containing acid fast bacilli resisted 1,000,000 M.L.D. while those with plain vaccine were resistant to only a 100 M.L.D. dose of the virus. The duration of resistance was also prolonged by the use of these adjuvants. The results with respect to antibody formation in the rabbit and ferret also indicated a potentiating effect.

Henle *et al.* (66) immunized human beings against influenza virus with the aid of water/oil emulsion omitting acid fast bacilli. In the control groups there was no added effect on repeated intramuscular injections of vaccine; i.e., one injection of allantoic fluid vaccine gave as good antibody response as three injections at weekly intervals. Although at two weeks after vaccination the antibody levels were increasingly higher with increase in the dose of antigen injected, the difference was slight in three months and by the end of six months all groups were practically alike. Prota-

mine did not exert any adjuvant effect. Using Falba and paraffin oil to prepare vaccine in water/oil emulsion (no mycobacteria were used) and injecting eighty subjects, there was abscess formation in two persons. Practically all subjects in this series exceeded the protective "threshold antibody level" of 1:128 within two to three months, whereas in the controls injected with slightly larger amounts of vaccine in saline solution, only 70 per cent ever reached the protective level in two weeks, the time of maximal response. The antibody titrations during eighteen months following vaccination suggest that protection may be afforded for at least two winters following vaccination with vaccine in water/oil emulsion. The authors believe that the increased antigenic stimulus exerted by the antigen in the form of water/oil emulsion cannot be due solely to the protective action of paraffin oil upon the antigen, because repeated injections of the vaccine in saline solution do not produce as high antibody titers as one single injection of the antigen in water/oil emulsion.

Warren (67) found in mice that encephalitis virus vaccine in water/oil emulsion produced a higher degree of protection than the same vaccine in salt solution.

Immunization against Rickettsiae.—Epidemic and murine types of (yolk sac) typhus vaccines in water/oil emulsion produce in rabbits a higher degree and longer lasting immune response than the same vaccines in saline solution. The immune response was measured by complement fixing and neutralizing antibodies. There was a fifteen to thirtyfold difference in the peak titers (Personal communication from Dr. John P. Fox of the Rockefeller Foundation).

Immunization against malaria.—Immunization with killed malaria parasites in water/oil emulsion with and without killed tubercle bacilli was studied in ducks and monkeys (68, 69, 70). The following conclusions were reached. It is possible to immunize adult ducks against infection with *P. lophurae* by the intramuscular injection of formalin killed parasites combined with paraffin-oil containing killed tubercle bacilli and an emulsifying agent. When the injections are made into the subcutaneous fat instead of the muscles however, no protection results. Repeated intramuscular injections of killed *P. lophurae* suspended in saline solution immunize against the parasites, but the protection is less effective than immunization with the aid of the adjuvants. Rhesus monkeys

injected twice with formalin killed *P. knowlesi*, emulsified in paraffin oil containing killed tubercle bacilli, survived with parasitemia of low grade when infected with one thousand or more *P. knowlesi*. Control animals receiving the same infecting dose died with a high level of parasitemia. Injection of formalin killed parasites without these adjuvants failed to protect. Davies (71) found complement-fixing antibodies in monkeys immunized with killed parasites in saline or in water/oil emulsion containing killed *M. tuberculosis*. The titers were higher with the adjuvants.

The reduction of systemic effect of toxins in water/oil emulsion.—An interesting aspect of immunization with antigens in water/oil emulsion is the reduction of toxicity. It was found that mice will tolerate 100 although not 1,000 M.L.D. of tetanus toxin in water /oil emulsion (48). Halbert, Smolens & Mudd (72) found that in mice fifty to three hundred times as much of *Shiga* neurotoxin, tetanus and botulinus toxins may be injected. Of the paradysenteric Flexner somatic antigen was about ten times and of ricin about fifty times more can be given in water/oil emulsion than in aqueous solution. Along with increased tolerance, there was apparently no effect on the antigenicity of these substances, rather antibody formation was very much more abundant with the vaccines in water/oil emulsion. The antigenic action of dysentery vaccines in water/oil emulsion was superior to that of vaccines combined with alumina.

The mode of action of water/oil emulsions.—The mode of action of the water/oil emulsion with or without mycobacteria has not been entirely elucidated. It is very likely that the absorption, destruction and elimination of antigen in water/oil emulsion is retarded. The protective rôle of paraffin oil in relation to killed tubercle bacilli is evident in the observation that in a skin nodule produced with killed tubercle bacilli suspended in paraffin oil and removed one year after the injection, a large number of bacilli were present (34). Working with *Shigella* antigen Halbert, Mudd & Smolens (73) estimated the residual antigen in mice injected subcutaneously with the bacteria in water/oil emulsion. The material from the site of injection was introduced into mice whose antibody response was observed. In their careful study they found that the amount of vaccine deposited had diminished within two weeks, but that antigen was still demonstrable twenty-two weeks after injection.

While the protective action of paraffin oil is well established, other factors may play a rôle in intensifying and prolonging antibody formation. Paraffin oil is known to cause a cellular reaction characterized by large numbers of mononuclear cells, the occasional appearance of foreign body giant cells, lymphocytes, polynuclear leucocytes and also fibrosis. It is known to intensify also the formation of tubercles in the presence of tubercle bacilli or saprophytic acid fast bacilli (42). These inflammatory cells may be important for antibody formation and sensitization. Observations by Rist (74) and by Casals & Freund (35) may be of significance in shedding more light on the action of paraffin oil. It was found that when killed tubercle bacilli incorporated in oil are deposited in the subcutaneous tissue, tubercles will appear in the draining lymph nodes and also in the lungs, mainly in subpleural situations. The dispersing effect may be more apparent than real, for if small numbers of tubercle bacilli should leave the site of deposition without oil following an injection of killed tubercle bacilli in saline solution, they may be readily destroyed and thus escape detection. The multiple persisting foci may contribute to the antigenic stimulation. The experiments (75) showing that the removal of killed tubercle bacilli in oil from the site of injection after a few days does not materially alter antibody formation or sensitization, suggest that multiple foci operate.

With regard to the ingredients of the water/oil emulsions the following questions arise. What is the rôle of the oil and of the emulsifying agents? Is it necessary to have a water/oil emulsion or is an oil-in-water emulsion effective? The rôle of paraffin oil is indicated by the observation that when peanut oil is substituted for paraffin oil, antibody titers are hardly higher than when antigen is employed in saline (using killed typhoid bacilli as antigen); further, monkeys were not protected against malaria when peanut oil instead of paraffin oil was used in the vaccine. The emulsifying agents insure the distribution of the antigen in the oil. They themselves may protect the antigens. Experiments made with oil-in-water emulsions suggest that the kind of emulsion used determines its effectiveness. The emulsions were prepared with Tween 80,[3] a highly active surface detergent, and paraffin oil, yielding an oil-in-water emulsion. Using this emulsion there was no adjuvant effect with respect to antibody formation.

[3] Atlas Powder Co., Wilmington, Delaware.

The possibility of incorporating antigens in the dry state directly in paraffin oil needs to be investigated. It was pointed out before that the water/oil emulsion exerts two effects: It increases and prolongs antibody formation. It has no or little effect on sensitization. Mycobacteria added to the emulsion also have two additional effects; they increase antibody formation and alter sensitization. This latter action on the sensitization seems to be the more significant one. It was found by Dienes *et al.* that when egg albumin is injected into a tuberculous lesion or when horse serum in water/oil emulsion containing killed mycobacteria is injected subcutaneously (44) the tuberculin type in addition to the anaphylactic type of sensitization to the proteins develops.

It would be of great interest to identify the fraction or fractions which exert the adjuvant effect of mycobacteria and to know whether the effect could be produced without sensitizing to tuberculin.

The type of sensitization to egg albumin or other proteins seems to be determined by the cellular reaction to tubercle bacilli. The cellular reaction caused by killed mycobacteria in paraffin oil is essentially similar to that caused by living tubercle bacilli (40, 41). Burnet (76) suggests the following hypothesis to explain the influence of tubercle on sensitization:

"to account for the difference between these sensitizing antibodies and the standard type, the following may be suggested: the histiocytes surrounding a subacute bacterial lesion are phagocytic and capable of producing antibody, but they are not so specialized for this function as the reticulo-endothelial cells of the major antibody-producing organs. The range of antigens with which they can react is probably limited, and does not include the type-specific pneumococcal polysaccharides. The antibody they produce is very much more readily removed from the circulation than normal antibody, and becomes fixed primarily on capillary endothelium, later perhaps in tissue cells throughout the body."

Inhibition of Experimental Drug Allergy and Anaphylaxis by Prior Feeding of the Sensitizing Agent

Chase (77) recently described a most interesting and puzzling response to the allergenic chemical 2:4 dinitrochlorobenzene. A solution of this compound in olive oil was fed to guinea pigs daily for six days; several such courses of feeding were given. After a period of rest the animals and control guinea pigs were given intracutaneous sensitizing injections. While the controls became highly sensitized, the experimental animals either did not become sensi-

tive or their sensitization was of low grade. The protection against sensitization lasted for several months after feeding. Chase also established that the action of feeding in inhibiting sensitization is specific. The resistant and the control groups behaved the same way when an unrelated substance, o-chlorobenzoyl chloride, was used for active sensitization. Feeding of the allergen to already sensitized animals has no effect on already established sensitization. An observation by Sulzberger (78) on inhibiting sensitization to neoarsphenamine may have a similar mechanism. He found that a single intravenous injection of this substance usually prevents subsequent sensitization by intracutaneous injections of the drug.

Picrylchloride is capable of inducing in guinea pigs both contact type of allergic dermatitis and anaphylactic hypersensitiveness. Chase (54) found that both types of sensitization can be inhibited at the same time by prior feeding of picrylchloride. Thus there is evidence that feeding is effective not only in the special field of allergy represented by contact dermatitis, but also in the anaphylactic type characterized by the well-defined role of serum antibodies.

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MEDICAL AND EPIDEMIOLOGICAL ASPECTS OF ENTERIC INFECTION

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This short account of the state of our knowledge of *Salmonella* and *Shigella* infections is prepared for—*sit venia verbo*—the consumer rather than the producer of investigational data. Emphasis, then, is placed on the basic aspects of our problem. References are primarily selected from the viewpoint of giving guidance to the numerous original papers. A more detailed account and more numerous references may be found for *Shigellae* in (1 to 4) and for *Salmonella* in (5, 6, 7).

Some Basic Bacteriological and Immunological Considerations

Details on the bacteriological properties of *Shigellae* and *Salmonellae* can be found in many textbooks [as for instance (8)] and in the references given in the preceding paragraph. The distinction of genera within the family and the further subdivision of the genera into species is essentially based on differences in metabolic properties. These evolved from the efforts of two generations of microbiologists to obtain means of distinguishing the pathogenic organisms from each other and from the numerous related commensals. It is well to realize that like all living matter, *Enterobacteriaceae* retain a degree of plasticity and variability. An increasing number of intergrading forms is becoming known (9, 10, 11). In contrast to our plentiful information concerning properties of minor biological importance, we are most ignorant about the mechanisms of many of the basic functions of these organisms, and particularly those relating to their behavior in the host.

The immunological differentiation of *Shigellae* and *Salmonellae* is essentially referable to two classes of substances. The first one is the somatic (complete or 0) antigenic complex, the constituents of which are carbohydrates, lipid-like substances and a polypeptide [see data in (1, 12 to 15)]. Of these, the carbohydrate moiety is the determinant of specificity. Secondly, there is a protein (13) called H or flagellar antigen. This is derived from the flagellae or, if

Pijper (16) is correct, from the envelope substance that presents itself in the form of flagellae. This antigen is lacking in nonmotile *Enterobacteriaceae*, as in *Shigellae*.

The components which are biologically most important in the microbial body, the protoplasm and nuclear substances, are not prominently involved in the antibody response of the host or of the animal injected with live or killed microorganisms. These substances are, however, not entirely devoid of antigenicity (13, 17).

Of the two major antigenic components, the flagellar antigen is of practical interest essentially because of its diagnostic value. It provides an extremely sensitive basis for distinguishing bacterial individualities.

The somatic antigen, however, is of biological importance beyond its differential value. First, it is (with one exception which will be discussed in a subsequent section) the only toxic constituent of *Enterobacteriaceae* (1, 2, 14, 15). This toxicity is not of a high degree, being on the order of a few mg. for rabbits, of 1 mg. for guinea pigs, and of 0.1 mg. for mice. Thus in this respect it can not compare with true bacterial toxins. The latter also differ from the somatic antigens in being liberated into the surrounding medium and in possessing an antigenicity different from that of the bacterial body substance. The confusing term "endotoxin" still substitutes for somatic antigens in publications and textbooks as a remnant from the time when the substrate of the poisonous effect of *Enterobacteriaceae* was unknown (1, 18). It is likely that the various effects caused by the somatic antigen in animals are dominated by its action upon the suprarenal (14, 19) and possibly the thyroid gland (20). The syndrome observed in experimental animals resembles in several respects that seen in the human being with infections of the type of enteric fever and severe dysentery.

The somatic antigen makes the microörganisms less accessible to phagocytosis, and it also impedes emigration of leukocytes from capillaries (21). It is possible, but not certain that the complete antigen is responsible for the "tumor-hemorrhagic" effect of Zahl & Hutner [references in (20)].

This agent of toxicity, the somatic antigen, is present in all *Enterobacteriaceae* regardless of their pathogenicity (14). Thus, obviously the somatic antigen does not explain the phenomena of pathogenicity nor the multiplicity of clinical disease caused by *Enterobacteriaceae*.

Toxicity of the antigen is a property of the entire complex. No single one of the known constituents is toxic (12, 14, 15). From certain indications in the fractionation of the somatic antigen it has been suggested that the toxicity may be located in an unknown (fourth) component (12).

The second fact which makes the somatic antigen so important is that acquired resistance to the bacterium is correlated with the antibody versus this antigen. Experimental animals may be actively or passively protected against infection with *Enterobacteriaceae* according to the specificity relations of their somatic antigens (22 to 25). The protective capacity of an immune serum can be removed by absorption with the proper complete antigen (25, 26). In man, vaccination with whole bacterial cells leads to the production of protective antibodies of the same type (22, 23, 27, 28). In the case of vaccination with the *Salmonellae*, as for instance typhoid vaccine, there is good evidence that such vaccination is reasonably (29, 30), though by no means completely (31, 32), protective against infection.

In general, differentiation by immunological technique fits well into the frame of classification by cultural properties. In many cases serological typing gives information well beyond that obtainable by bacteriological methods. However, this does not mean that immunological identification could supplant the classical bacteriological techniques. The reason for this is that serological specificities are not absolute. As could be predicted from the structure of the carbohydrate moiety responsible for their specificities, similar or related antigens may well occur in more than one microorganism, and particularly in other *Enterobacteriaceae*. A number of cases of nonpathogenic microorganisms with antigens similar to that of pathogenic ones have already become known (2, 5, 9, 10, 11, 33, 34, 35). It should, however, be noted that such "unspecific" reactions are rare and errors can largely be avoided by a proper combination of bacteriological and serological techniques.

The typical somatic antigen is quite heat stable. However, there are antigens (14) of essentially similar constitution which are damaged by heat (36 to 40), the best known of which is the Vi antigen [Felix (36)] of the typhoid bacillus [see p. 322].

Under proper conditions, small numbers of many *Shigellae* or *Salmonellae* cause generalized infections in the mouse (mucine method) (41, 42) and the chick embryo (43 to 46). This is of par-

ticular importance for work with *Shigellae* since monkeys are the only naturally susceptible animals (47). But for *Salmonellae* too, test animals which are easily handled and obtainable in large numbers are of considerable usefulness.

SHIGELLAE

The following species are usually distinguished more by tradition than from cogent reasons (2):

Sh. dysenteriae ("the Shiga bacillus")
Sh. ambigua ("The Schmitz bacillus")
The *Shigellae* of the Large-Sachs group (five or possibly seven serological types) (48, 49)
Sh. paradysenteriae ("the flexner bacillus"—nineteen serological types) (50, 51, 52)
Sh. sonnei ("the Sonne bacillus") (53)
Sh. alkalescens (54)

In addition there are several individual serological forms of which only one or a few strains have been isolated, and which cannot be discussed here [for data see (2)]. *Sh. dispar* [see (55)] presents one of the "border line" cases of pathogenicity for the human being which will be mentioned in the closing section of this paper. Only about ten of the thirty known serological types of the genus are found with frequency and are thus of major practical concern.

Epidemiological aspects of shigellosis.—It has been suggested that all infections caused by *Shigellae* be called "shigellosis" (56, 57). A general acceptance of this term would have the great advantage of directing attention to the causative agent of the preponderance of diarrhoeal disease. This, indeed, would signify progress. At present, only a minute fraction of diarrhoeal disease is etiologically diagnosed (2, 57). In the United States in 1944 thirty-eight thousand cases were reported (58). The true incidence can only be guessed. It has been estimated from mortality figures that in twenty-one states of the Union where dysentery is a reportable disease, there were at least seven hundred thousand cases for the years from 1934 to 1937 (1). The United States Army recently gave a figure of 523,449 known diarrhoeal infections during the war years (59). Though most of these cases are not serious clinically, they maintain the chain of infection and recruit the carriers. They account for the fact that in many states of this country intestinal

infections are still in the foreground of every statistical study of infantile mortality (1, 2, 57). It is estimated that in the years from 1935 to 1940 over ten thousand children under two years of age died of "diarrhoea" annually in the United States (60). In Cincinnati, between 1939 and 1941, there were three times as many deaths from "diarrhoea" as from diphtheria, scarlet fever and pertussis combined (60).

Dysentery remains the cause of frequent epidemics of serious character in homes and institutions. Infection rates in homes and institutions may reach figures of more than 100 per cent per year (57). Food handlers are, of course, prominent as originators of outbreaks, and the carriers in this occupational group equal that in the general population, if no precautions are taken. Water borne infection is a less serious danger, though such epidemics have been described [see (61)]. For additional sources of statistical data the reader is referred to (1, 2, 4, 57). The benefits which we derive from our high standards of sanitation and nutrition are probably not so much in minimizing the number of infections as in lowering the numbers of organisms ingested in the average case and in the maintenance of high general resistance. Where these favorable circumstances are lacking, dysentery retains in its age old position, mitigated only by the current usefulness of sulfonamides. It is a part of the widespread delusion concerning the actual facts to call dysentery a tropical disease. It is one only insofar as in many tropical countries the nutritional and general sanitary standards are low.

Chronic ulcerative colitis develops as a sequela of dysentery in perhaps 10 per cent of all cases (4, 62).

As to the prevalence of species of *Shigella*, enough data have been collected to give a general picture. Some characteristic data are presented in Table I, which is an excerpt of data given in (1, 2). The Shiga bacillus is rare except in the Middle East and in India; possibly also in Poland. (Of course, figures from large parts of the earth, as for instance China and Russia, are entirely lacking.) *Sh. ambigua* is found the world over, though nowhere very frequently. Neither are the types of the Large-Sachs group found with any frequency, though the rather scanty data indicate that they are world wide in distribution. *Sh. alkalescens* is found everywhere (2, 3); however, it is probably not always and under all circumstances significantly pathogenic. Thus, it is not too rarely

found as a companion of other pathogens, and it is distinctly more often found associated with infections of the urinary tract than other *Shigellae* or *Salmonellae* (2, 3).

The Flexner bacillus and *Sh. sonnei* together represent everywhere the bulk of *Shigella* infections [(1, 2) also references in Table I]. In some places, the Sonne bacillus is found in 75 per cent or over of all cases. A closer analysis shows that such reports concern the surveys which include the less severe diarrhoeas. Milder cases are

TABLE I

RECENT STATISTICAL DATA ON PER CENT INCIDENCE OF *Shigella* SPECIES

Year	Country and Region	Specimens	Shiga	Ambigua	Flexner	Sonne	Alkalescens	Others or unclassified	Reference
1936–39	U.S.A., New Mexico	2081	0	x	80.9	19.1	x	x	(63)
1937–40	U.S.A., Mississippi	760	2.2	1.7	80.1	15.1	x	1.1	(64)
1943–44	U.S.A., South-Eastern States	2113	0	1.2	33.4	62.0	3.3	0.1	(65)
1939	Scotland (Glasgow)	608	0	0	56.1	43.9	x	x	(66)
1942	Germany	7071	0.9	x	13.2	85.9	x	x	(67)
1943–44	Algeria*	476	2.3	2.6	72.3	15.5	1.4	5.9†	(68)
1940–43	British Middle East Forces	61509	18.9	6.7	61.6	7.4	x	5.5‡	(69)
1938–40	India	9777	10.7	7.1	65.1	16.4	x	0.7§	(49)

* U. S. Army laboratory.
† 2.4% *Sh. dispar.*
‡ 3.3% mannitol +and 2.2% mannitol negative *Shigellae.*
§ Large-Sachs group.
x = not mentioned in report.

more often caused by the Sonne bacillus than by other *Shigellae.* However, it would be erroneous to conclude from this that infections with *Sh. sonnei* are always light (1).

Since the group of Flexner bacilli (*Sh. paradysenteriae*) causes the majority of reported cases of bacillary dysentery, information concerning the serology of this species is of obvious interest. The now classical work of Andrewes and Inman covering about two-thirds of all strains still stands, but it has been complemented by the investigations of Boyd (50, 69) to a point where the larger majority of all occurring strains can be classified. On the basis of reinvestigations in this country (51, 52), a system of designation has been suggested (52) which distinguishes nineteen types by

their major antigens (symbolized by Roman numerals) [see also (2)].

Properly absorbed sera offer a means of rapid identification of types by simple slide agglutination (51, 52, 69, 71). Such typing sera were used on a large scale in the laboratories of the American and British forces (68 to 71), and are now also available for civilian use. The same method can be applied for the identification of the other species and types of *Shigella*.

From the still incomplete information at hand, it appears to be certain that only a restricted number of Flexner types is found with frequency, namely I, I.III, II, III, IV, and VI. Some characteristic data are given in Table II.

Our views concerning the spread of the infection have been clarified by epidemiological investigations in Denmark (76) and in this country (57, 63, 77). In contrast to the situation with the *Salmonellae*, man is the only primary source of infection. The acutely or chronically ill are not the main sources of danger to the community. Convalescent and healthy carriers (these terms coalesce, because of the numerous undiagnosed or mild cases) are very frequent, much more so than formerly surmised. Again we have to refer for additional data to (1 to 4).

A carrier rate of one to twenty or more per thousand (63) of any population provides an abundant source of infection at any time. Human hands are the most important means of contamination of food. Where human dejecta are not shielded from flies, these may play a role in carrying bacilli from feces to accessible food (78). The carrier and his hands are the focal point of all efforts to break the chain of infection.

The prevailing mode of infection explains why present methods of sanitation, though effective in preventing mass catastrophes, fail to eliminate permanently the sources of infection. It also makes clear why dysentery is an inseparable companion of such social upheavals as war. That our armies and fleets were able to move all over the world without catastrophic impediment by dysentery was a feat which did not find due recognition. How much of this was due to applied sanitation, and how much to the use of sulfonamides and the susceptibility of the organisms to these drugs (of which more will be said later on) is difficult to gauge.

Clinical aspects of shigellosis.—*Shigellae* do not, as a rule, cause

TABLE II

EXEMPLIFYING RECENT STATISTICAL DATA ON THE PER CENT INCIDENCE OF TYPES OF *Sh. paradysenteriae* (FLEXNER)

Year	Country and Region	Specimens	Types*						
			I	I.III	II	II.VII	III	VII	VIII
1943–44	U.S.A., South-Eastern States	709	3.1	x	26.9		3.2	10.1	2.1
1943–44	U.S.A., California	161	35.4	x	0.6	x	5.6	x	x
1944	U.S.A., Michigan and New Mexico	142	46.5	x	25.4	x	7.0	0	2.8
1946	Brazil, Rio de Janeiro	200†	7.5	16.0	34.5	2.0	19.0	1.0	3.0
1944–45	France‡	183	4.8	x	3.3	26.8	6.6	6.6	0
1932–35	India	4800	77						

* Nomenclature according to (2, 52).
† 13 cultures autoagglutinable.
‡ American troops and German P.O.W.'s.
x Not mentioned in report.

generalized infections. The characteristic localization is in the muscular layer of the colon, from where it may affect the adjacent mucosa with subsequent ulceration (4).

Dysenteric infections, particularly the lighter ones, may lack the leading symptom of diarrhoea, an additional reason for preferring the term shigellosis. [For a catalogue of the disguises under which shigellosis may hide see (4).]

The genus *Shigella* contains the only species of *Enterobacteriaceae* known to produce a true exotoxin, namely *Sh. dysenteriae*, the *Shiga* bacillus. [For a similar German assertion concerning *Sh. ambigua* (79), the writer found no confirmation either in his own experience or in the literature.] The product of the Shiga bacillus conforms with the requirements of a microbial toxin in every respect, including that of high potency and distinct antigenicity. Antitoxin neutralizes the toxin in high multiples and has been demonstrated in infected human beings as well as in immunized animals [see (1, 2)]. However, there is no evidence that the Shiga toxin plays a significant part in producing the pathology of dysentery. Neurological symptoms dominate the picture following experimental application of Shiga toxin to such an extent that it is often designated as neurotoxin. These symptoms do not form a typical part of the syndrome of human dysentery. The symp-

TABLE II (*Continued*)

Types*										etousae	Reference
III.IV	IV	V	VI	IX	X	XI	XII	XIII	XIV		
x	1.6	0.1	52.5	0	0.2	0	0	0	0.1	x	(65)
x	18.7	5.6	8.7	4.4	1.9	1.9	7.4	2.4	7.4	x	(72)
x	9.2	2.8	5.6	0	0.7	0	0	0	0	x	(73)
0.5	2.0	1.5	3.5	0	2.0	5.0	0	0	0.5	x	(74)
0	2.6	1.7	46.7	0.5	0	0	0	0	0	0.5	(75)
x	4	2.5	7.5	4	1.5	1.5	0.2	0.3	1.5	x	(50)

tomatology of human dysentery in cases of infection with Shiga's bacillus is the same as in those caused by the other *Shigellae* which do not produce an exotoxin. Also, Shiga antitoxin is not protective against experimental infection, whereas the antibody versus somatic antigen is protective in very small quantities (28, 80, 81).

As noted in the preceding section, the symptoms caused by the somatic antigen in experimental animals are so similar to those seen in clinical "toxic" dysentery, that these substances are likely to play a role in the pathology of the human disease. Direct evidence is, of course, not easily obtainable.

Man is not highly susceptible to the infection as such. From semiquantitative experiments in human volunteers in this country with virulent bacilli of Flexner type II (82) and from German data on *Sh. sonnei* (83), it appears that 10^9 microorganisms represent approximately a 50 per cent infective dose in healthy adults.

Malnutrition and other debilitating factors may well reduce resistance. Children, and particularly infants, are without doubt more severely affected than adults as regards both clinical disease and mortality. It would not be justified to conclude from the severity of infection that children are also more susceptible in terms of infective dose.

Prevention of Shigellosis.—Much remains to be done in regard to the indoctrination of those who are in daily contact with the immediate problems of dysenteric infection. We have to depend

on the alertness of the practicing physician and of the health officer for the recognition of the carrier and the "innocuous" intestinal disturbance if we want to reduce the sources of danger to the individual and the community. The slow but steady rise in numbers of diarrhoeal infections reported in this country and the corresponding substitution in these reports of the diagnosis "unclassified" by "bacillary" testifies that some improvement is being achieved in this all important educational aspect of the problem (4, 58).

We are not in possession of an effective specific prophylactic agent. Vaccination has been tried many times, but reports on the results of such measures remain contradictory. This is to be expected in view of the fact that protection is type specific, and that until recently there was no way of knowing whether the antigens present in a vaccine corresponded with the type of prevalent infection. In this connection it is pertinent that exact information concerning immunity after clinical infection is so lacunary that we cannot even answer the question whether or not infection leaves homologous resistance.

During the recent war, only the German army employed a (polyvalent) vaccine (67), and this was abandoned in 1942 for reasons unknown (84). Neither the results of somewhat amateurish efforts to obtain information about the response to this vaccine (85), nor knowledge about the prevalence of dysentery in the German army (4, 86) speaks for the effectiveness of the preparation. A not inconsiderable effort was made in this country to explore the possibilities of prophylactic vaccination. As a result, the response of human beings to vaccination in the form of a considerable outpouring of protective antibody was established (12, 25, 27, 87). But it is not obvious that such antibody is correlated with clinical protection. As a matter of fact, a well controlled and rather large scale experiment in volunteers who had received Flexner type II vaccine in an amount known to lead to considerable production of circulating antibody, gave no evidence of clinical protection to oral infection with this organism in a dose which caused slightly more than 50 per cent infection in untreated controls (82).

This outcome is in rather puzzling contrast to the results of experimentation in mice (24, 25, 28, 88) and particularly in monkeys (47). This writer feels that we have no right to abandon attempts to obtain an effective vaccine. The currently discernible approaches to this goal are discussed in (2).

Therapy of Shigellosis.—The success of sulfonamides in the treatment of dysentery is common knowledge [references in (2, 4)]. Unfortunately, the good tidings already have become subject to considerable restriction. Experimental resistance of *Shigellae* to sulfonamides has been observed for some time (89). Recent reports have told of an increasing incidence of shigellosis clinically resistant to sulfonamide therapy, with corresponding findings of *in vitro* fastness of the strains isolated from such cases (90, 91). Thus, our Pacific Fleet had been trailed for a considerable length of time by Flexner type III infections. Eventually a large outbreak occurred which proved to be due to sulfonamide resistant organisms of this type (91). This, by the way, exemplifies how the precise identification of *Shigellae* gives the epidemiologist a secure basis for his observations.

We may in the future revert to a position where the therapeutic armamentarium is not much different from that available fifty years ago. The presently available antibiotics do not hold out a large promise for dysentery. A large field is still open for therapeutic research. This may well include a thorough examination of the therapeutic possibilities of bacteriophages, about which much has been written and preciously little is known. Phage therapy is rather widely employed abroad. For instance, the Russians are using it on a rather large scale and seem to like the results (94). But there also objective data are not available. The clinical experiment with a German preparation collected by the British after El Alamein (86) had a negative result (it was *in vitro* also rather ineffective). However, the experimental results in animals (92) are encouraging enough to invite the search for an effective preparation and its clinical evaluation according to objective standards. [For a summary of our present knowledge see (92)]. The collection of data on the relation of bacteriophages, which do not attack all *Shigellae* uniformly (93), remains to be done as a prerequisite for a rational approach to the problem.

Salmonella

The Serological Differentiation of Salmonellae.—For the classification of *Salmonellae*, the schema of Kauffmann & White (5) has been internationally accepted. The somatic antigens serve for primary differentiation into groups. The antigens are designated by Roman numerals, and the groups by capital letters. Thus, the group designation corresponds with the types of *Shigellae* pre-

sented before. For instance group A, of which the sole member is *S. paratyphi A*, has the antigens symbolized by the letters I, II, and XII. Group B, which comprises among many others *S. paratyphi B*, and *S. typhi murium*, has the antigens I, IV, V, and XII. Group C_1, which includes, for instance, *S. paratyphi C*, *S. cholerae suis*, and *S. orianenburg*, has antigens VI and VII. Group C_2, of which a member is *S. newport*, has antigens VI and VIII. Of group D, the most important member is the typhoid bacillus (*S. typhi*); it also contains *S. enteriditis* and *S. pullorum*. Its antigens are I, IX, and XII. There are three groups in which all have the antigen III, but which differ in the additional somatic antigens, and which are designated as E_1, E_2, and E_3. And there are finally a number of rare types with somatic antigens different from those of the main groups which are listed as group F (5, 6, 7, 112), but which really ought to form independent groups as proposed by Barnes (95).

Within the groups further differentiation into what is often called types is obtained by determination of the specific phase flagellar antigens. Of these so many have become known that the original designation by small letters had to be supplemented by z with subscripts; other flagellar antigens (group phase) are designated separately by arabic numerals for reasons which cannot be detailed here [see (5, 6, 7, 112)].

The total number of *Salmonellae* distinguishable by serological technique is now above two hundred. Lack of space does not permit giving a complete list here. Tabulations will be found in (5, 6, 7, 112). Tabulation of newly described types will be found in (95) and (112).

The array of over two hundred types looks rather formidable. However, the number of types of high incidence is much smaller as illustrated in Table III, which gives all the types found with a frequency above one per cent in five statistical reports.

The complete determination of the antigenic pattern of a given strain has become a rather specialized job. For this purpose, several *Salmonella* Centers have come into being as, for instance, that connected with Beth Israel Hospital in New York (96) and that at the Agricultural Experiment Station in Lexington, Kentucky (7, 97). These in turn are in contact with the International *Salmonella* Center in Copenhagen. A number of state health laboratories also provide facilities for *Salmonella* typing [see (98)]. If the principles discussed in the previous paragraphs are under-

TABLE III

Representative Data on Incidence of the More Prevalent *Salmonella* Types in Man

Material from			U.S.A. (96)	U.S.A. (97)	U.S.A.* (98)	England and Wales (8)	Italy† (99)
Total Number of Strains			2229	532	358	1506‡	892
Group	Type	Antigenic formula					
A	Paratyphi A	I, II, XII: a	(16)	7	0	—‡	14
B	Paratyphi B	I, IV, V, XII: b—1, 2 . . .	140	132	57	—‡	87
	Typhi murium	I, IV, V, XII: i—1, 2, 3 . . .	675	63	53	672	51
	Derby	I, IV, XII: f, g, z_8	54				
	Bredeney	I, IV, XXVII, XII: l, v—1, 7 . . .		8			
	San diego	IV, V, XII: e, h—e, n, z_{15} . . .		13			
C_1	Cholerae suis	VI, VII: c—1, 5 . . .	144	30	5	22	20
	Oranienburg	VI, VII: m, t . . .	150	18	11	76¶	187
	Montevideo	VI, VII: g, m, s . . .	126	24	15	37¶	119
	Bareilly	VI, VII: y—1, 5 . . .	43	13	7		
	Thompson	VI, VII: k—1, 5 . . .				142	
	Tennessee	VI, VII: z_{29}					51
C_2	Newport	VI, VIII: e, h—1, 2, 3 . . .	238	53	15	159	
	Bovis morbificans	VI, VIII: r—1, 5				23	
D	Typhi	I, IX, XII, Vi: d	122	23	146	—‡	218
	Enteritidis	I, IX, XII: g, o, m . . .	44	12	4	172	
	Panama	I, IX, XII: l, v—1, 3, 4, 5 . . .	92	27	8		
	Dublin	I, IX, XII: g, p				19	
	Napoli	I, IX, XII: l, z_{15}—e, n, x . . .					31
E	Give	III, X, XXVI: l, v—1, 7 . . .	30		4		20
	Anatum	III, X, XXVI: e, h—1, 4, 6 . . .		12	4		
	Meleagridis	III, X, XXVI: e, h—l, w . . .				19¶	43
	Newington	III, XV: e, h—1, 6 . . .	91	37§			
	Senftenberg	I, III, XIX: g, s, t . . .		11			16
			1949	483	329	1341	857
Per cent of total *Salmonella* isolations			87.4	90.8	91.9	89.0	96.1
Number of types with an incidence of more than 1 per cent of all isolations			13	16	12	10	12

* Connecticut.
† Allied armies and civilians.
‡ Cases of food poisoning only—thus exclusive of typhoid, paratyphoid A and B.
§ 31 carriers.
¶ Believed to be introduced from U.S.A. by spray-dried eggs.
( ) Not in totals.

stood, the recipient of a type diagnosis will, with the help of one of the available tabulations, be able to take full advantage of this information.

For the routine laboratory, a set of sera is now generally available which allows the identification of all important groups of *Salmonella*, as suggested for instance in (96, 100). (The set is supplemented by sera which allow the differentiation of *S. typhi* from other members of Group D.)

The Vi Antigen.—At this point, a few words are warranted concerning the heat labile somatic antigen of *Salmonellae*, particularly of *S. typhi*. This antigen was first observed by Felix (36) when studying the so called inagglutinability of certain typhoid strains. It was ascertained that the substance interfering with the agglutination of typhoid strains by O sera possesses an antigenicity of its own. Subsequently the antigen was found to be a complex similar to other somatic antigens except that it is unstable when heated and that it is less toxic (14). It is found in practically all freshly isolated strains, but may be lost on culture in artificial media. Strains having Vi antigen are less susceptible to phagocytosis [see (5, 18)]. For this and other reasons, Felix assumed that this antigen has something to do with the virulence of the typhoid bacillus. Definite evidence in this respect has not been forthcoming. Moreover, the mere fact that strains of *E. coli* have been found (5) that posess a Vi antigen undistinguishable from that of *S. typhi* makes it unlikely that the Vi antigen has any great importance for invasiveness or other properties pertaining to "virulence." A considerable amount of experimental work on the Vi antigen has been summarized in (5, 18, 37). From the results of this work only two points can be mentioned here: Vi antigen is found in other *Salmonellae* as for instance in the (rare) *S. paratyphi C.* Antibody to Vi antigen is found in carriers with enough regularity that testing of the serum of suspects is of some practical importance (101, 102).

Bacteriophage Typing of S. Typhi.—The lysis of *S. typhi* by bacteriophage is in many cases related to the Vi antigen. However, not every bacteriophage lysing the Vi form of typhoid bacilli attacks every strain. A closer investigation of this situation led Craigie [see (103)] to the discovery that typhoid bacilli can be grouped according to sensitivity to certain phages. Specificities are thus revealed which are not detectable by immune analysis. Subtyping of *S. typhi* on this basis has proven to be a very sensitive tool

for epidemiological follow-up and similar work (103, 104, 105).

S. paratyphi B has also been claimed to be susceptible to phage typing (106).

Epidemiological Significance of Salmonella Typing.—Salmonella typing has had an impact on epidemiology comparable with that of the serological analysis of the X group of pneumococci. This is well illustrated by Table III which gives the more important results from five statistical studies. Of particular interest is the high incidence of *Salmonellae* of groups C_1, C_2, and E, as *S. oranienburg*, *S. montevideo*, *S. newport*, and *S. newington*.

Examinations of "healthy" individuals are providing revealing figures on the incidence of symptomless carriers. Thus, 20 per cent of isolations in Lexington, Kentucky (97) came from carriers. Still we have no data on large-scale random examinations in populations. But it is becoming clear that human carriers are quite frequent and, therefore, one important source of infections.

Animals are, of course, known to be afflicted with a variety of *Salmonella* infections (107). And *Salmonellae* may be carried in apparently healthy animals. This is true not only of mammals and birds (108), but also of reptiles (109) and insects (110). Obviously, animals may be the source of human infection. Moreover, *Salmonellae* can be carried from animal to man by animal products, particularly those used for food.

A considerable percentage of the egg powder produced on a large scale during the war years was found to be contaminated with *Salmonellae*. It is likely that in this manner certain *Salmonella* types were introduced into England (8, 96, 111).

Inversely, the exploration of an outbreak will find some guidance by typing of the *Salmonella* involved. Some *Salmonellae* are able to infect nearly every animal species as, for instance, the ubiquitous *S. typhi murium*. Others show a surprising degree of species specificity or, as it has been termed, host adaptation [see (6)]. The best known examples are the human "specialists" *S. typhi* and *S. paratyphi A* and *B* which are for all practical purposes pathogenic for man only. The classical example of an animal *Salmonella* is *S. pullorum* which has developed specialization for the chicken and other *Galliformes*, and even its own peculiar mode of transfer (107). In Fig. 1, a schematic presentation of host adaptation is given.

Types like *S. typhi murium* are of world-wide distribution.

Others show an outspoken pattern of geographical distribution. Thus, *S. paratyphi A* is definitely rare in this country, but a frequent cause of enteric fever in the Middle East and the East, as indicated by war experience (32) and particularly by data in the Annual Reports of the Public Health Commissioner with the Government of India which were made available through the kindness of General Boyd. In the years from 1933 to 1941, 283 isolations of *S. paratyphi A* were made in India, in comparison with 529 of *S. typhi*, and only twenty-five of *S. paratyphi B*.

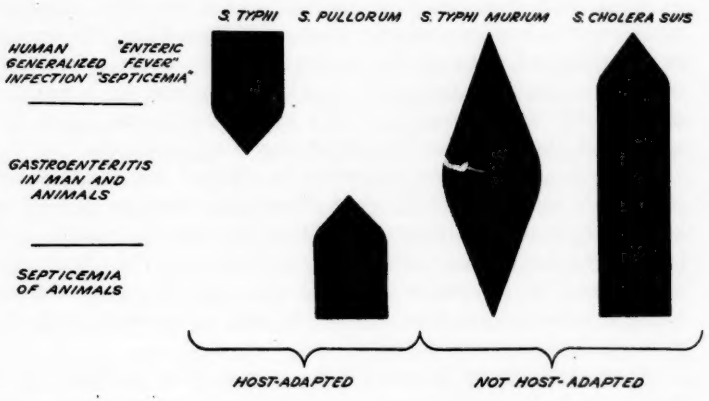

FIG. 1.—HOST ADAPTATION OF *Salmonellae*. SCHEMATIC PRESENTATION OF FOUR REPRESENTATIVE EXAMPLES.

Clinical Aspects of Salmonellosis.—It would falsify essential aspects of the clinical problems if the group of diseases caused by *Salmonellae* were considered as primarily located in the intestinal tract. The classical case of the salmonellosis (113, 114) is that of a generalized infection, namely that of typhoid fever. This human clinical picture has its close parallel in animal salmonellosis (107). There is the clinically important distinction between generalized infections of this kind that some lead to purulent localization (septicemic foci) (5, 96). In either case it is the blood (or the focus) from which the microorganism is most regularly isolated; eventually though, the causative agent reenters the intestinal lumen, often leading to a more or less characteristic localization in the

intestines on its way out. This process many years ago was aptly compared to the secretion of heavy metals (115). Where the strain involved is not of high invasiveness, the bacteria do not progress beyond the lymphatic glands draining the intestines [(116, 117) see also (6)]. And there may be cases where *Salmonellae* of low pathogenicity cause cursory symptoms merely by virtue of the toxic effect of the numerous organisms swallowed in food.

Obviously the degree of involvement of the host is the result of a complex interplay of factors, some dependent upon the microorganism, such as the infecting dose (118) and the mysterious thing called virulence (119), and others upon the host, as specific immune resistance or the factors that make for nonspecific resistance or lack of resistance. Such nonspecific host factors are, for instance, the state of nutrition, exhaustion, primary illness, and age (120, 121, 122). Infants are highly susceptible (96). Hereditary factors probably play a role as evidenced by extensive experimentation in animals (123, 124, 125). These studies clearly demonstrated that hereditary factors are involved in the resistance of mice, and went further to indicate that such factors may partially or predominantly be expressed as the ability to mobilize phagocytes (124, 125).

As to a specification of the term virulence there is no information available beyond the facts uncovered by Ørskov's experiments in mice (117) which showed that invasiveness, *ceteris paribus*, depends (*a*) upon the ability to penetrate the intestinal wall, and (*b*) that invasion progresses along the lymphatic channels. Microorganisms of low invasiveness may be stopped at the mesenteric lymph glands, whereas the more virulent ones proceed beyond this point to enter the blood stream. It is also pertinent that the typhoid bacillus at least propagates intracellularly both in the chick embryo and in man, that one of the preferred sites of multiplication is the leukocytes and that only the macrophages are able to disarm the invader (116, 126).

There is a restricted correlation between type of *Salmonella* and invasiveness. In general the human body is able to overcome infection with nonadapted *Salmonellae* better and quicker than that with the adapted *S. typhi* and *S. paratyphi A* and *B*. This generalization definitely does not exclude the occurrence of an occasional severe infection from nonadapted *Salmonellae*. There is a notable exception to this rule which concerns *S. cholerae suis*. This type is the most pernicious of all *Salmonellae* as witnessed by the high

percentage of septicemic infections and a lethality which may reach 20 per cent (6, 96, 99).

The diarrhoeic type of *Salmonella* infection is clinically not easily distinguished from that caused by *Shigellae*. However, it appears (58) that *Salmonellae* do not evoke the ulcer formation characteristic of shigellosis. Though *Salmonellae* have variously been found in a considerable percentage of diarrhoeic infections (127) they are as a rule, not a frequent causative agent for this kind of disease (57, 145).

The lethality of a given outbreak will depend largely on the percentage of generalized infections, which in turn is influenced by the virulence of the organism as well as age, nutritional state and other factors influencing the population affected. One may suspect that the number of ingested organisms also plays a role [see (118, 119)].

As the conditions which make for high unspecific resistance are favorable in this country, it is not surprising that fatality rates in the United States in recent years are often lower than those found in older statistics [(96), see however (128)]. The more impressive are the exceptional death rates for *S. cholerae suis* infections (6, 96, 99).

Therapy of Salmonellosis.—*Salmonella* infections are sometimes favorably influenced by sulfonamide treatment, but more often this class of therapeutic agents is of little effect. This is particularly true for the more serious generalized forms of "typhoid fever" (6). This disappointing outcome of clinical experience was the more unexpected as both in *in vitro* and in the experimental animal (43) *S. typhi* is susceptible to sulfonamides.

Penicillin is generally not effective against gram negative microorganisms, and what little is known about streptomycin does not sound very encouraging (129). It appears that this antibiotic temporarily suppresses enteric growth. However, *Salmonellae* reappear when streptomycin is discontinued. At least this was found true in five infants harboring *S. typhi murium* (130). Data on the effect of large doses of streptomycin are not available at the time this report is written.

Under these circumstances one may regret that hyperimmune serum is not available in this country. The British experience with serum therapy in typhoid fever [(131, 132, 133), see also (18)] seems encouraging enough. A combination of therapeutic agents

(as for instance sulfonamides with penicillin or serum) appears to be a definite possibility as indicated by (134, 135) and also by animal experimentation (43). The circumstance that immune protection depends on antibody versus the group specific (somatic) antigens indicates, that if immune sera versus the groups B, C_1, and D were available they would satisfy the needs of the great majority of cases where immunetherapy may be life saving.

Craigie's method of bacteriophage typing of *S. typhi* invited investigation of the therapeutic effect of "fitted" phages in typhoid fever. Experimental (136, 137) and preliminary clinical reports (138) are favorable and certainly merit further attention.

Immune Prophylaxis in Salmonellosis.—A reinvestigation of the antigenicity of the strains routinely used for the preparation of typhoid and "triple" vaccine (42) resulted in the introduction of strains rich in somatic antigen (42, 139) and measures for the controlled maintenance of antigenicity. This work resulted also in valuable data on the levels of circulating mouse protective antibody in vaccinated persons (42, 140).

In realization of the importance of the C_1 group the British army developed a quadruple vaccine containing typhoid and paratyphoid A, B, and C bacilli. A similar preparation is not available in this country. From the data on the incidence of *Salmonellae* in this country, it would not be necessary to employ a quadruple antigen, as the British had to use in view of the need of protecting personnel likely to see service in the Orient. Substitution of a properly selected strain of the group C_1 for the present para A antigen would probably be satisfactory.

The demonstration [(141), see also (18)] of the advantages of intracutaneous immunization has not led to a widespread use of this method. The United States army, however, has made revaccination by the intradermal route permissible.

The Question of the Pathogenicity of Enterobacteriaceae other than Shigellae or Salmonellae

The literature contains a considerable number of reports on outbreaks of diarrhoeic disease where none of the known pathogenic *Shigellae* or *Salmonellae* could be isolated. In a number of severe outbreaks amongst the new born in hospital wards a filterable agent has been found which recently was shown to infect calves (142) and to grow in the chick embryo (143). In adults,

viral enteritis has been definitely demonstrated in one mild outbreak (144).

In a much greater number of such outbreaks suspicion has been directed against enteric bacteria that are usually nonpathogenic, such as members of the colon group, *Proteus*, and *Ps. aeruginosa* [numerous references in (2)]. The main argument in all these cases, besides the negative one of absence of known pathogens, is the demonstration of an identical organism in many individuals during the same outbreak. It would certainly be unjustified to deny *a priori* the possibility that strains or maybe certain types of nonpathogenic *Enterobacteriaceae* may develop disease producing ability. However, it is difficult to obtain definite evidence, because (*a*) the microorganisms in question are ubiquitous inhabitants of the normal enteric tract; (*b*) enteric disorders are known to favor a more or less temporary shift of the commensal flora to uncommon forms (2, 145); (*c*) commensal bacteria spread by exactly the same mechanisms as pathogens, and it is therefore only to be expected that a given group, as for instance the inhabitants of an institution, should possess a similar intestinal flora.

At the present time, then, it may be wise to reserve judgment on this question until further data are available. The same is true with regard to the question of the pathogenicity of a microörganism which for bacteriological as well as serological reasons is closely related to the pathogenic *Shigellae;* namely, *Sh. dispar* (2, 3, 55).

It may not be superfluous to point out that pathogenicity as discussed here has to be distinguished from two sets of circumstances where bacteria are involved, namely the growth of huge numbers of bacteria in food so that enough toxic material—be it bacterial matter or products of bacterial metabolism—is swallowed to cause an acute enteric upset (food poisoning in the most strict sense of the term); and bacterial invasion in individuals of lowered resistance as, for instance, those afflicted by malignant tumors.

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LEDERLE LABORATORIES DIVISION
AMERICAN CYANAMID COMPANY
PEARL RIVER, NEW YORK

THE RICKETTSIAE

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Rickettsiae

The rickettsiae and the diseases they cause have been intensively studied during the past six years. The recent work was stimulated by the probability that many troops would be exposed to certain of the rickettsial diseases during the war. Another strong stimulus was the development of methods for the cultivation of large numbers of rickettsiae in the yolk sacs of hens' fertile eggs. Until 1940–41 the investigations of this group or organisms had been primarily concerned with the diseases per se, the epidemiology, vector transmission, and reservoirs. Zinsser and his group and others had done some work on serological relationships but were handicapped by the fact that production of adequate quantities of suspensions rich in rickettsiae was difficult if not almost impossible. Much had been done on the relationships of the rickettsiae to the *Proteus* X strains although no very satisfactory explanation of the Weil-Felix test had been forth coming. The differentiation of the various rickettsial agents depended almost entirely upon the disease picture produced in animals and the results of cross-immunity and neutralization tests. These at best were frequently somewhat crude and demonstrated only major differences. Only Rocky Mountain spotted fever vaccine had been produced in sufficient quantity to be of practical significance. It was derived from the tissues of infected ticks and its manufacture so tedious and costly that the supply was very limited. Many had described methods for the preparation of vaccines against typhus fever but none of these had been definitely proven to be of value, or they involved such difficult and delicate procedures that they were not considered practical.

This, then, was the status of the rickettsial field when several methods were described for the cultivation of large numbers of rickettsiae, and the war focused attention upon this group of diseases.

CULTIVATION

In hens' fertile eggs.—Cox (1) in 1938 opened the way to more definitive studies of the rickettsiae by describing their successful cultivation in the yolk sac of the developing chick embryo. Although certain of the rickettsiae will grow in other parts of the fertile egg the most abundant growth occurs in the yolk-sac membrane. When tissue impression smears are stained by Machiavello's method (2) the rickettsiae often can be seen in great profusion. Some of the organisms can be seen within cells but most are lying free among the cell debris. It is presumed that they were growing within cells which ruptured in the preparation of the smear. In the past, great stress has been laid upon the location of the rickettsiae within the parasitized cell, *Rickettsia rickettsi* invading the nucleus, and *R. prowazeki* the cytoplasm. This is not of great practical significance when the rickettsiae are cultivated in yolk-sac membranes.

All of the rickettsiae, pathogenic for man, that have been thoroughly studied, grow from moderate to well in yolk-sac membranes, these include: *R. rickettsi*, *R. conori*, *R. akari* (3), *R. prowazeki*, *R. mooseri*, *R. orientalis*, and *R. burneti*. The technique is simple and lends itself well to the cultivation of sufficient quantities of organisms for the preparation of antigens.

In the lungs of animals.—Castaneda (4) in 1938 showed that large numbers of *R. mooseri* developed in the lungs of rats following intranasal inoculation. Usually several passages are necessary before the strain becomes well adapted to growth in the lungs. Durand & Sparrow (5) adapted Castaneda's technique to *R. prowazeki* and showed that this organism could be cultivated in the lungs of mice. Fulton & Joyner (6) cultivated *R. orientalis* in the lungs of rodents, mice and cotton rats.

This technic has been used extensively in various parts of the world for several immunological procedures. Although it is a simple method, it has several disadvantages when compared to cultivation of the rickettsiae in fertile hens' eggs. In most localities animals are more expensive, the intranasal inoculation of animals with viable rickettsiae is an extremely dangerous procedure, and finally the constant possibility of strain contamination with naturally occurring rodent diseases must be considered.

Growth

Although very little is known concerning the essential growth factors of the rickettsiae several studies have cast some light upon this subject. Since the rickettsiae grow only in the presence of living cells studies on growth are necessarily complicated. It was shown years ago that x-rayed rats (500R) or benzol poisoned rats, and vitamin C deficient guinea pigs were not only more susceptible but that fairly large numbers of rickettsiae could be cultivated by intraperitoneal injection. Rats with riboflavin deficiency were shown by Pinkerton & Bessy (7) to yield similar results. Snyder, Maier & Anderson (8) opened up new fields of study when they found that *p*-aminobenzoic acid could prevent deaths in animals infected with typhus fever. Hamilton, Plotz & Smadel (9) carried this finding over to the cultivation of *R. prowazeki* in eggs. They found that *p*-aminobenzoic acid inhibited the growth of the rickettsiae. Greiff, Pinkerton & Moragues (10) about the same time found that *p*-aminobenzoic acid was an active inhibitor while sodium fluoride apparently would increase the growth of *R. prowazeki* in eggs.

The explanation of the activity of *p*-aminobenzoic acid is not clear at the present time. It is not rickettsiocidal but apparently acts in a competitive manner with some enzyme system essential to proliferation of the rickettsiae, although it may act by stimulation of the metabolic activity of the host's cells.

Serologic Tests and Preparation of Antigens

As has been stated, the cultivation of rickettsiae in the yolk sac and mouse lung has made possible considerable immunological investigation. Most of the ordinary serological procedures have been applied to the study of rickettsiae.

Thus the complement-fixation test was applied by Castaneda (11), by Bengtson (12, 13), and by Plotz (14). Most of the tests are now performed by the technic of Bengtson (13) or that of Plotz (14). The complement-fixation test has been used routinely for years as a diagnostic aid in rickettsial diseases. The technic of Bengtson has the advantage of using a more easily prepared and less expensive antigen, while the technic of Plotz gives more specific results between closely related strains.

Yolk-sac and mouse lung cultures of typhus fever rickettsiae

have been used by a number of investigators as a source of antigen for agglutination tests (15 to 18), and for a precipitin test (19).

It was observed by Gildemeister & Haagen (20) that suspensions of yolk sacs heavily infected with *R. mooseri* injected intraperitoneally in mice caused rapid toxic deaths, and that immune serums neutralized the toxic effect. Bengtson, Topping & Henderson (21) showed that suspensions of yolk sacs heavily infected with *R. prowazeki* were also lethal for mice, and Henderson & Topping (22) worked out a neutralization test for both *R. prowazeki* and *R. mooseri*, whereby mixtures of toxic material and dilutions of serum are mixed, incubated, and inoculated intravenously in mice. This neutralization test is used in this country to test the potency of typhus vaccines.

It should be pointed out that this toxic effect of *R. prowazeki* and *R. mooseri* has been produced only by the injection of large numbers of viable rickettsiae. It would seem that removing the rickettsiae by centrifugation also removes toxicity, and agents such as formalin and ether, which kill the rickettsiae, also destroy the toxic effect. It has been our experience that calling this toxic activity a "toxin" causes considerable confusion, since to so many people the word "toxin" refers to a specific chemical substance. It, therefore, appears wise at this time to use words such as "toxic effect," "toxic material," or "toxic substance," since it is entirely possible that the mice are killed by metabolic activity of the viable rickettsiae.

Another type of neutralization test was described by van den Ende & Mills (23) who made use of the fact that strains of *R. mooseri* and *R. prowazeki* adapted to mouse lungs by serial intranasal passage could cause discrete focal lesions in the lungs. These pulmonary lesions could be counted. Incubation of the rickettsial inoculum with specific immune serum resulted in reduction of the number of focal pulmonary lesions produced by intranasal inoculation.

Craigie *et al.* (24) have described a modification of the Giroud skin test in rabbits. The lesions produced are specifically altered by immune serum, and their methods allow quantitative estimation of the antibody content.

The preparation of antigens satisfactory for serologic tests and for vaccination has been the subject of considerable study. The preparation of antigens illustrates the properties of the rickettsiae,

so this aspect will be discussed in more detail than the actual technics involved in performing the tests.

Since the yolk sac is the most convenient culture medium, many of the methods for preparation of antigens use yolk sac as the starting material. Ordinarily a 10 to 20 per cent yolk suspension in physiological saline is first made. This preparation contains rickettsiae and tissue particles in suspension, soluble substances such as protein, and lipids in emulsion. Fifty per cent or more of the solids in yolk sac may be ether-soluble. The presence of much lipid in emulsion complicates the problem of obtaining satisfactory antigens.

Craigie (25) first suggested the use of ethyl ether in this connection, and he pointed out that when suspensions of yolk sac in saline were shaken with ether, much insoluble material was carried to the interphase (emulsion, or middle layer), whereas the rickettsiae of typhus fever remained in the aqueous phase.

Shortly after this Topping & Shear (26) demonstrated that ether treatment resulted in a release of considerable "soluble" antigen, and Donovick & Wyckoff (27) found that a more potent vaccine resulted. As a result ether extraction was used in the manufacture of the typhus vaccines in this country and in Canada for the immunization of military personnel.

The behavior of various strains of rickettsiae when treated with ether offers a basis for their practical classification (28). When a 10 per cent suspension of yolk sac in physiologic saline is shaken with one or two volumes of ethyl ether and allowed to stand, three layers appear; the upper, clear yellow, ether phase; a middle emulsion or interphase; and a lower, fairly clear, aqueous phase. *R. orientalis* is attracted to the interphase, while the others, *R. prowazeki*, *mooseri*, *rickettsii*, *conori*, *akari*, and *burneti* remain in the aqueous phase. It has been mentioned that some rickettsiae release a soluble antigen when treated with ether. Thus if the aqueous phase is drawn off and angle-centrifuged at 4,000 r.p.m., for one hour, the rickettsiae will be sedimented, and if soluble antigen has been released it may be detected in relatively high titer in the supernatent fluid. With the exception of *R. burneti* all of the rickettsiae remaining in the aqueous phase, release a soluble antigen.

One may prepare complement-fixation test antigen for all the rickettsiae except *R. orientalis* by the above method, namely by shaking an infected yolk-sac suspension with ether. However, in

the case of *R. rickettsii* because of poor yolk-sac growth and *R. burneti* because it does not release soluble antigen, some concentration is frequently necessary. Such a concentration may be achieved by centrifuging the saline suspension before it is ether extracted. Considerable purification is also effected by this step.

In the case of *R. orientalis*, because of its attraction to the interphase, a further modification is necessary in order to have an antigen satisfactory for complement fixation. One such modification consists of ether extracting yolk sacs which have been ground without the addition of saline, and then adding saline after the ether extraction. Much insoluble material may then be removed by centrifugation (28). In another modification by Wolfe *et al.* (29) yolk-sac suspensions are dried in vacuum before being ether extracted and finally rehydrated.

A method for the preparation of rickettsial antigens from yolk sacs without the use of ether has also been developed. In this procedure centrifugal washing of yolk sac suspensions is done in phosphate buffer at a pH of 7.6 to 7.7. This treatment removes the lipids in emulsion and the soluble materials and a final absorption with Celite selectively removes tissues, leaving the rickettsiae in a nearly pure state. This method is applicable to all rickettsiae which may be easily sedimented, that is, all except *R. orientalis* (30).

Immunological Relationships Among the Rickettsiae

It has become possible to discuss the immunological relationships among the rickettsiae now isolated. Enough cross-reaction exists under certain conditions to indicate the presence of broad groups.

Typhus-spotted fever group.—Cross-reaction is frequent between *R. prowazeki* and *R. mooseri*. Cross-reactions among *R. rickettsii*, *R. conori*, and *R. akari* are also common. Ordinarily cross-reactions between these two sub-groups is not frequent enough to be bothersome in diagnostic work, but Plotz *et al.* (31) showed that fairly frequent cross-reaction is seen with *R. rickettsii* convalescent serums and typhus antigens in the neutralization test of Henderson and Topping. This cross-reaction is also seen in the complement-fixation test but so rarely that it does not interfere with its usefulness in routine diagnostic work. Further common properties of the rickettsiae of the typhus-spotted fever group are the release of soluble antigen on treatment with ether, and the stimulation of

antibody formation against *Proteus* OX19 (except *R. akari*).

The relationship between *R. prowazeki* and *R. mooseri* has been studied rather extensively. These organisms were once thought to be antigenically identical because of the solid cross-immunity to infection that occurs in guinea pigs after an infection produced by the other strain. The two antigens may be easily distinguished serologically by the higher titers that occur in the homologous reaction (*prowazeki-prowazeki* or *mooseri-mooseri*) than in the heterologous reaction (*prowazeki-mooseri*). Craigie *et al.* (24), Fulton & Begg (17), and Shepard (32) have investigated this problem in some detail. [See also Wishart & Malcomson (33)]. From this work it seems clear that absorption of a typhus immune serum by the heterologous strain removes the heterologous antibody, leaving a serum that reacts only with the homologous antigen. It does not appear possible to remove homologous antibody by absorption with excess heterologous antigen. These observations are interpreted to mean that both *R. mooseri* and *R. prowazeki* contain specific antigenic groupings for themselves, as well as groupings common to both.

The work of Craigie *et al.* showed that the antigens of these two strains were at least partially labile to heat at about 60°C. They and others found that heat-treated antigen lost its strain specificity and that it was no longer capable of stimulating immunity to infection or to the toxic substance. When tested by the complement-fixation test, heat-treated antigen absorbed the antibody causing cross-reaction. However, when tested by agglutination and precipitin tests the cross-reacting antibody was not removed. Fulton & Begg, and Shepard showed that heat-treated antigen had a certain degree of new specificity. Thus the serum of animals immunized with heat-treated antigen would not react with native antigen, although it would react with heat-treated antigen (17, 30, 32). However, the new specificity was not complete because reactions did occur between antiserum to native antigen and heat-treated antigen.

The relationship of typhus and *Proteus* OX19 antigens was also investigated (17, 32) with the conclusion that *Proteus* OX19 antigen was more closely related to the heat-altered antigen than it was to the native antigen. Typhus antigens do not always stimulate the formation of *Proteus* OX19 antibodies. Following immunization with typhus vaccine there is little or no rise in *Proteus* OX19

antibodies (34), and vaccinated persons undergoing attacks of typhus fever show diminished formation of *Proteus* OX19 antibodies (35). The most satisfactory interpretation of these observations seems to be that the antigens of typhus fever rickettsiae are closely enough related to those of *Proteus* OX19 so that under certain circumstances cross-reaction is marked, while under other circumstances no cross-reaction is seen.

Fortunately, for the protection of troops against epidemic typhus by vaccination, strains of *R. prowazeki* in various parts of the world appear to be identical. Thus Topping, Bengtson & Henderson (36) found no difference in the response of guinea pigs, receiving vaccine made of three different strains of *R. prowazeki.* Later unpublished work by the same authors and by Craigie brought additional strains under study with the same result, and further indicated that strains of *R. mooseri* from different isolations were homogeneous.

The apparent serological homogeneity of separate isolations of *R. prowazeki* on one hand and *R. mooseri* on the other has another possible application which should be pointed out. It arises from the fact that the classification of a newly isolated strain as *R. prowazeki* or *R. mooseri* is now on a more definite basis, since it may now be done clearly and quickly by serological methods. The homogeneity of *R. prowazeki* and of *R. mooseri* and the absence of strains of intermediate characteristics would make it unlikely that strains of one type are arising from the other type, so that the epidemiological origin of each is distinct. Considerable argument has occurred in the past as to the origin of strains in epidemics, and the difficulty in differentiating *R. mooseri* and *R. prowazeki* probably added to the confusion.

Cohen & Chargaff (37) have studied the substances present in fractions of preparations of yolk sacs infected with *R. prowazeki.* The immunological significance of the fractions was not thoroughly considered, but it appears that some of the fractions they prepared correspond to the soluble antigen mentioned above. Their work is complicated by the difficulty of obtaining pure rickettsial material from yolk-sac suspensions. The reader should refer to the original article for details of this work.

Shepard & Wyckoff (38) have employed electron microscopy and the metal shadowing technic to investigate the release of soluble antigen from typhus rickettsiae. They found capsular material to

be present on the surface of rickettsiae and that ether extraction of rickettsial suspensions resulted in a stripping off and breaking up of the capsular material into droplets of a size range of 40–100 mμ. The rickettsial origin of the droplets was shown by their agglutination with specific antiserum.

In the spotted fever group (*R. rickettsii*, *R. conori*, and *R. akari*) cross-reactions have been reported as common by Plotz, Reagan & Wertman (39) and Huebner, Stamps & Armstrong (3). Plotz states that if the soluble antigen is removed, the specificity of antigens of *R. rickettsii* and *R. conori* is considerably increased so that cross-reactions between the two are much less important.

Tsutsugamushi (*scrub-typhus*).—*R. orientalis* is the only rickettsia thus far observed which is attracted to the ether-water interphase. It is also the only one giving rise to *Proteus* OXK antibody formation. Cross-reactions between *R. orientalis* and other rickettsiae are rare. Unlike the strains of *R. prowazeki*, for example, the strains of *R. orientalis* are not homogeneous. In this laboratory, various strains have also shown marked variation in virulence for mice and guinea pigs, and in adaptability to growth in eggs. From a practical standpoint, a more important aspect of the variation is in the complement-fixation test as Bengston (40) reported.

In order to detect antibodies in a case of naturally occurring tsutsugamushi disease it may be necessary to use at least two well-chosen strains. For this purpose the Karp strain and the Gilliam strain appear to be well suited (41). However, strain specificity is not complete, and Topping (42) showed that in guinea pigs solid cross immunity to infection occurred after recovery from any of the four strains tested (the Karp & Gilliam strains were included).

The antigenic pecularity of *R. orientalis* is also reflected in the difficulty of preparing vaccines against it. Methods applicable to the other rickettsiae are not satisfactory here. So far clear protection has been evidenced only following intraperitoneal vaccination and intraperitoneal challenge (6, 43, 44), and subcutaneous vaccination has not protected against intraperitoneal challenge. However, the protection afforded by intraperitoneal vaccination appears to be specific (6).

Q fever.—*R. burneti* has been studied recently in some detail because of the discovery of its occurrence in large numbers outside of Australia. Earlier reports of comparisons of strains had failed to show significant differences, and all strains isolated to date do give

solid cross-immunity to infection. However, two strains recently isolated in Italy and in Greece showed considerable differences in serologic tests as shown by Topping, Shepard & Huebner (45) and by Robbins *et al.* (46). This difference was not reflected in the usual way as it is between *R. prowazeki* and *R. mooseri* and among strains of *R. orientalis*, but it appeared chiefly as a marked difference in sensitivity of the antigens in the complement-fixation test and in the agglutination test. Thus, the two strains mentioned, and especially the Italian (Henzerling) strain, gave much higher antibody titers than the other strains with serums of guinea pigs recovered from infections with all strains of Q fever tested. It appears that only such a strain as the Italian (Henzerling) is satisfactory as an antigen in a diagnostic test, since if such a strain is not used, many cases of Q fever will not be diagnosed serologically. The serological diagnosis of Q fever is at present the only satisfactory one for routine work, since the clinical picture is such that Q fever may be confused with a number of other diseases.

It is possible that differences in specificity of the strains also exist, but it seems more wise for the present to emphasize the great difference in sensitivity, since this is clear-cut.

Immunization

As mentioned before, the only rickettsial vaccine available in sufficient quantity to be of practical value was that for Rocky Mountain spotted fever. Since 1926 it had been prepared from phenolized infected tick tissues. Following the cultivation of the rickettsiae in fertile eggs and in the lungs of rodents these methods were utilized immediately in the preparation of vaccines. Cox & Bell (47) described a method for the preparation of epidemic and murine typhus vaccine. This was later shown by Topping & Bengtson (48) to have rather poor antigenic capacity in animals. Craigie (25) suggested the use of ethyl ether in processing but Topping & Shear (26) showed that a soluble antigen was released by the other treatment and hence was being discarded in the method originally described by Craigie. Topping (49) outlined a method that was used by most of the manufacturers of typhus vaccine in the United States during the war. Craigie reported a method retaining the soluble antigen. Groupe, Nigg & MacFarlane (50) have described in detail methods used by one of the large commercial producers. The methods using infected yolk-sac membranes are also applica-

ble to the preparation of Rocky Mountain spotted fever vaccine and this is now available commercially. Vaccine for murine typhus can also be made by the same method and at least one producer has been licensed for production and sale of the vaccine.

With the advent of vaccines prepared from fertile eggs and proposals for their widespread use in troops, interest was taken in their allergenic properties. Thus Coulson & Stevens (51) showed that these vaccines had allergenic and anaphylactogenic properties in animals. Although there can be no doubt that these vaccines carry with them the inherent danger of anaphylactic reactions shared by all proteins, the experience acquired by the U. S. Army during the war indicates the rarity of severe and fatal reactions. Approximately 6,000,000–8,000,000 troops received between 15,000,000 and 30,000,000 individual doses of typhus vaccine with only two fatalities reported as attributable to its use.

Typhus vaccines prepared from the lungs of rodents and larger animals after the method described by Castaneda (4) were used in Europe during the war. The German army apparently used several types of vaccine, from yolk-sac membrane, from infected lice after the method originally described by Weigl (52), and from the lungs of mice.

Results

The field evaluation of epidemic typhus vaccine has proved to be very difficult. In the laboratory, evidence has been collected regarding the immunizing power of present day vaccines. In vaccinated animals there can be no doubt of complete protection against massive challenge doses. Topping, Henderson & Bengtson (53) showed, in studies involving some 400 humans given a primary immunization of two doses a week apart, that both neutralizing and complement-fixing antibody had fallen considerably in five months and had all but disappeared at the end of nine months. At the end of the nine months period a single booster dose of 1 ml. was administered with a remarkable rise in both the neutralizing and complement-fixing antibody. During the subsequent observation period of nine months, the complement-fixing antibody gradually decreased until it was no longer demonstrable by the end of the study. In contrast the neutralizing antibody titer remained high during the entire period of observation. It was remarked that these titers were comparable to those observed in convalescents from actual cases of epidemic typhus fever. These studies pointed

out the importance of booster doses in the development and persistence of demonstrable antibody to *R. prowazeki.*

Gold & Fitzpatrick (54) reported on the lessened severity of typhus fever contracted by vaccinated laboratory personnel and Topping (35) compared a group of vaccinated and unvaccinated personnel accidentally infected in the laboratory. This study indicated a considerable lessening of severity of the infection in the vaccinated group with resultant reduction in symptoms and signs as well as loss of time from work. Van den Ende *et al.* (55) reported several infections in laboratory personnel in England where the evidence of reduction in severity of illness in the vaccinated was not as clear.

Ecke & Gilliam (56) analyzed the experience among vaccinated (with from one to three doses of vaccine) and unvaccinated employees of the Cairo Fever Hospital. The conclusions were that here too there was a reduction in the severity of the disease following vaccination with even one dose of vaccine. Ding (57) reported from Germany on experiments involving the direct challenge with infectious material of vaccinated and unvaccinated humans. The conclusions were that there was definite protection in the vaccinated group.

The U. S. Army experience involving several million men vaccinated against epidemic typhus fever and where many had intimate exposure to the disease is worthy of note. The Surgeon General of the U. S. Army, Norman T. Kirk, has reported that there were a few cases of very mild typhus fever in army personnel but that no deaths were recorded as being due to this disease.

The only other rickettsial vaccine where reports of the results in humans are available is Rocky Mountain spotted fever. Parker (58) has reviewed the experience with this material over the past fifteen years. He states

"The vaccine has definite immunizing value. The degree of protection conferred varies with the individual, the relative virulence of the infecting strain, the number of successive years that vaccine has been administered, and perhaps to some extent with the age of the person vaccinated."

Treatment of Disease

Immune sera.—Hyperimmune rabbit serum was developed by Topping (59) against Rocky Mountain spotted fever, and against typhus fever by Kurotchkin, Van der Scheer, & Wyckoff (60). In animals (59) hyperimmune sera for Rocky Mountain spotted fever

was shown to prevent deaths when administered subcutaneously after the onset of symptoms. Topping (61) in 1943 reported the use of hyperimmune rabbit serum in the treatment of human cases of Rocky Mountain spotted fever. Data was presented which indicated that the observed fatality rate in those cases treated before the third day of rash was considerably lower than that indicated from past experience with patients receiving no serum. Of the fifty-two cases treated in this group there were only two deaths, both in males 66 and 72 years of age, respectively, or a fatality rate of 3.8 per cent as compared to the expected rate of approximately 18.8 per cent. Anigstein & Bader (62) found that very small doses of hyperimmune rabbit serum administered intradermally as late as forty-eight hours following an intradermal inoculation of virus would still prevent the disease in guinea pigs. If the amount of injected serum was small the guinea pigs were subsequently found to be immune. If the amount of serum administered was large the guinea pigs did not subsequently develop immunity.

Hyperimmune sera for epidemic typhus fever was administered under controlled conditions to human cases of the disease in the Cairo Fever Hospital by Yeomans, Snyder & Gilliam (63). It was felt that definite therapeutic effects resulted, although the series of patients was small.

A hyperimmune rabbit serum for tsutsugamushi disease (scrub typhus) was prepared by Topping (64). These antibodies can be passively transferred to mice previously infected with certain lethal doses of yolk-sac material from the homologous strain. Death can be prevented in these infected mice, when treated with the serum, after a lapse of 72 to 168 hours from the time of infection. This serum has not yet been given a therapeutic trial in human cases of tsutsugamushi disease.

Chemotherapeutic Agents

Snyder, Maier & Anderson's (8) report that *p*-aminobenzoic acid had activity against *R. prowazeki* in animals stimulated considerable research on this and other chemotherapeutic agents. Thus Yeomans *et al.* (65) reported on the clinical results observed in twenty patients with louse-borne typhus fever who were treated with *p*-aminobenzoic acid in Egypt. They concluded that large doses of *p*-aminobenzoic acid exerted a definite beneficial effect on the course of louse borne typhus if treatment is started in the first week of illness.

Snyder *et al.* (66, 67) found that *p*-aminobenzoic acid had beneficial activity in the treatment of gerbilles infected with *R. orientalis* (tsutsugamushi disease). Tierney (68) reported that of eighteen human cases of the disease treated with *p*-aminobenzoic acid no deaths occurred while in sixteen untreated cases there were three deaths. A large dosage of the drug was given and high levels were obtained in the blood. Toxic effects were observed in patients receiving the higher dosages. The optimum blood level was finally considered to be 30 to 60 mg. per ml.

Anigstein & Bader (69) reported encouraging results in the treatment with *p*-aminobenzoic acid of guinea pigs infected with Rocky Mountain spotted fever. Rose, Duane & Fishel (70) followed shortly with a favorable report of the treatment of a human case of the disease. Flinn *et al.* (71) reported on the treatment of ten human cases of the disease. Nine were under forty and all recovered; however, the only one over forty died. Topping (72) in analyzing the experience over a ten-year period in four states has shown that the fatality rate in Rocky Mountain spotted fever is greatly influenced by age. Under forty years the rate is only 13 per cent but over forty it is about 40 per cent. Flinn *et al.* indicated that there was dramatic clinical improvement shortly after treatment began, this together with the experimental work certainly suggests therapeutic benefit from the drug, but reduction in fatality rates has not as yet been proven.

Both methylene blue (73) and toluidin blue (74) have shown therapeutic activity in experimental rickettsial infectious in animals. Methylene blue in a treatment trial of human cases of tsutsugamushi disease was found to be too toxic in the necessary dosage. Smaller doses seemed to be of little or no value.

Investigations of chemotherapeutic agents for typhus fever were undertaken by members of the British Medical Research Councils' staff at the National Institute for Medical Research, London (75). Over two hundred drugs were tested for activity against experimental typhus infection in mice. Two of the compounds were selected for special investigation, as they were found to have definite action in preventing the development of the disease in mice. These are the substances designated V 147 and V 186, which are *p*-sulphonamidobenzamidine hydrochloride and *p*-sulphonamidobenzamidoxime hydrochloride respectively. Although they are sulphonamides in the strict chemical sense of the term, their activity is not inhibited by *p*-aminobenzoic acid, and there-

fore, their mode of action, like their range of activity, is distinct from that group of drugs commonly known in clinical medicine as "sulphonamides." The activity of these new drugs, in a field where the latter are ineffective, is of considerable importance, and a study of their mode of action may lead to a better understanding of the relationships between chemical constitution and biological activity in this field. In view of the activity shown, and as the two drugs were found to have no excessive toxicity for animals or for human volunteers, it was considered justifiable to try them in cases of typhus in man.

"Unfortunately, under the conditions in which the trials with V 147 and V 186 were carried out, treatment with the drugs did not prove to be effective. Cases were rarely available for treatment early in the disease, at the stage at which, in accordance with the results obtained with the drugs in experimental animals, therapeutic value might have been expected to be shown. When used at the later stage at which most patients came under treatment, there were indications that the drugs, in some instances, may have accentuated the known liability to kidney damage in severe cases of typhus."

The study of these various chemotherapeutic agents in the rickettsial infections do indicate, however, new substances with activity based on a different mode of action. Further studies are needed to elucidate this action as well as to determine more active and less toxic drugs.

Antibiotics.—Grieff & Pinkerton (76) reported that penicillin inhibited the growth of typhus rickettsiae in the yolk sac of fertile eggs. Moragues, Pinkerton & Grieff (77) also reported that penicillin was an effective therapeutic agent when administered to dba mice infected with murine typhus. However, no reports have been seen which indicate that penicillin is an effective therapeutic agent in human cases of typhus fever, or for that matter, of Rocky Mountain spotted fever. Further, penicillin has been tried in several cases of Q fever without any evidence of beneficial results (78).

Smadel, Jackson & Gauld (79) have reported on the rickettsiastatic effect of streptomycin in experimental infections. Using the technique previously described (9) they found that 1 to 2 mgs. of streptomycin per egg delayed the death of the embryo when the yolk sacs were infected with *R. prowazeki*, *R. mooseri*, or *R. rickettsii*. When the dosage was increased to 10 mg. of streptomycin per egg the effect was greatly enhanced. However, dosages as high as 20 mg. per egg had no noticeable effect on *R. orientalis*. It was further noted that there was an apparent synergistic effect when administered with *p*-aminobenzoic acid.

SUMMARY

The investigations of the rickettsiae have progressed significantly during the past few years. Since these organisms require the living cell for proliferation and yet are large enough to be stained and seen with the optical microscope, they lend themselves well to fundamental studies. It is now realized that these organisms represent an effective biological tool that may be utilized for studies of the basic requirements of that class requiring living cell for proliferation. The immunological and immunochemical characteristics of the rickettsiae may well aid in the development of concepts of more general application. Methods and technics have been and probably will be further developed which may prove to have wider utilization than indicated by their present use in studies on the rickettsiae.

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RESPIRATORY VIRUSES*

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The present discussion proposes only to emphasize phases of the study of certain viruses of respiratory disease which are of interest in characterizing the agents and in seeking to fill gaps in our knowledge of the biology of the respective diseases. The subjects chosen are fruitful fields for continued investigation from many angles. In most instances the data are in a stage of evolution and the evaluations must, therefore, be limited except as to indicate trends of thought which may be applicable to virus research in general. The bibliography is a selected one, limited in many instances to papers dealing most extensively with the subject or with original statements.

Influenza Virus

Reservoir.—The recurrent epidemics of influenza A and B continue the question of how influenza virus persists in the intervals. In the past few years it has been adequately demonstrated that both influenza A and influenza B occur sporadically (1), giving weight to the thesis that they represent at present endemic infections building to epidemic proportions of various extent, at intervals of two to three years in the case of influenza A; at intervals of four to five years with influenza B. But the seasonal upswings of influenza A or B are much less uniform in time than those of other diseases such as diphtheria, measles, or chickenpox. Between times scattered cases and small outbreaks develop but the general antibody levels of the population tends to decline. Influenza B has been notable for its scattered persistence, as in 1945 when it was a year long outbreak (2).

To date persistent human carriers have not been reported and the immunological data tend to weigh against their being common. During epidemic periods, however, a high incidence of inapparent infections is encountered, indicating that the virus may establish itself and stimulate antibody production without eliciting signifi-

* The author wishes to acknowledge his appreciation of the assistance of Dr. J. J. Guilligan in the collection of material.

cant signs of illness and suggesting that the same circumstances could obtain undetected in interepidemic periods. Furthermore, active infection can occur without significantly altering an antibody level. The variations observed in the response of different individuals to a common infection is sufficient indication that concealed infection can take place. Moreover, the observed variation in pathogenicity and of epidemicity of different strains of virus are readily in keeping with the idea of Andrewes (3) that a degraded virus may be the persisting form. Hare & Mackenzie (4) have recently reviewed certain instances of infection imported to secluded populations by apparently well individuals—an epidemiological commonplace. But it is not clear that the agents are degraded rather than that the resistance of the bearer is high. In this case the infection may be latent but masked only by technical deficiences. The same criticism can obviously be applied to our ignorance of the amount of continued infection which prevails, a difficulty to a large extent eliminated in measles because the stigmata are readily recognizable. The possibility that influenza virus may be maintained in man in a latent, dormant state, thence aroused by physiological insult, is, however, obviously suggested by Shope's studies (5) of swine influenza. Shope has presented data indicating that swine influenza virus escapes from the hog's lung in the lung worm, persists in the life cycle of that parasite through the earth worm and its return to the lung of a new hog. During its residence in the worm outside the hog, virus is not detectable by infectious activity or by serological means. In this masked state the virus resides in the worm in the lung of the hog until the injection of certain disturbing substances such as calcium chloride or the toxic *Hemophilus influenzae suis* disturbs the equilibrium, the virus is activated, enters pulmonary tissues and excites the virus disease. During the warmer seasons of the year the provocation of disease is not effected by these various influences but cold of itself does not activate the virus. Earthworms collected from the soil of a hog ranch in Iowa where swine influenza had been present were fed to normal hogs in New Jersey in June; provocation aroused the virus infection in the autumn at the time of its usual seasonal occurrence. Thus, while Shope has not encountered carriers of the usual, recognizable virus, in its occult form the agent may persist from one season to the next. The activation of virus and the institution of patent disease leading to epidemic preva-

lence would then be referable to constitutional and environmental factors rather than to the concept of a constant circulation which becomes enhanced as a sufficient nucleus of susceptibles is accumulated. But Glover (6) has told the writer that in the British Isles carriers of pathogenic swine influenza virus are not infrequent among older hogs. Blakemore & Gledhill (7) indicate the same probability and report, in addition, the recovery of virus from the lung of a hog that had been sick eight weeks. Under such conditions the reservoir of virus could well be a patent one and the time of outbreaks in a herd would depend upon the collection of susceptibles.

These data invite further work in clarification of the maintenance of influenza viruses by the human population. Although there are no reported instances in which influenza viruses of both Types A and B have been recovered simultaneously from a single individual most observers have encountered individuals who have developed antibodies to both viruses on recovery from an acute illness. It has been commonly accepted that they represent examples of immunological spread in infection with one virus, anamnestic reactions. It is interesting, nevertheless, to speculate whether this phenomenon might constitute activation of a carrier or latent virus of one type during the course of infection with the other.

McKee & Hale (8) have recently demonstrated that influenza virus combined with immune serum in an over-neutralized mixture so as not to be recovered by dilution or by serial passages in mice, may be released by the addition of large amounts of inactive virus. Recognizing the fact that antibodies in the respiratory secretion could well conceal virus, the application of a similar procedure toward the detection of virus in the secretions of carriers is suggested.

A precise demonstration of the mechanisms through which the reservoir of the disease functions would constitute a great advance in constructing the biological pattern of influenza. The possibility that swine influenza could be transferred to man seems ever present and, in view of the characteristics of swine virus, that it has not been observed in the outbreaks of man, is somewhat surprising. There remains, too, the unexplored possibility that the ferret, mink, weasel or other members of that susceptible family could serve as sources of human infection. Ferrets (9) and swine (10)

have been found spontaneously infected with strains of influenza virus spreading in the human population, but this is the reverse of the question asked.

Hemagglutination.—Agglutination of chicken erythrocytes by influenza virus and its inhibition by immune serum were reported by Hirst (11). Later, McClelland & Hare (12) reported the same observations, independently made. They noted, in addition, that cells of humans and guinea pigs were agglutinated; those of rabbits, rats, mice, horses and sheep were not. They demonstrated further that the virus was adsorbed to the cells and that the agglutinating and infectious activity were adsorbed together.

These phenomena and the subsequent demonstration of elution of adsorbed virus (13) have contributed enormously to the study of influenza and the influenza viruses. The original isolations in the chick embryo of influenza virus (14, 15) from patients involved subsequent animal adaptation for identification. With the use of hemagglutination as an indicator the employment of eggs for primary isolation has been practicable, and intra-embryonic, amniotic and allantoic routes have all yielded a significant proportion of successful results (16, 17, 18).

Hemagglutination and its inhibition by immune serum (19) have facilitated immeasurably serological diagnosis and epidemiological studies. It has aided and, at times, exaggerated the appreciation of serological variations in strains of virus. The adsorption of virus by erythrocytes has served, as suggested by McClelland & Hare (12) in their original report, as a valuable method for concentration and purification of virus (20, 21, 22). It has led to the demonstration that other viruses, notably those of Newcastle disease (23) and mumps (24), have similar activities while those of vaccinia and ectromelia have a limited effect.

The precise nature of the reaction has not been demonstrated. Hirst (13) showed that for a period after its adsorption to the cell the virus remained attached, after which it gradually eluted. Nevertheless, adsorption was complete in one hour even at 4°C. and the rate of elution increased with temperature. The speeds differed between the PR8, Type A strain, and the Lee, Type B strain. When the virus had eluted from the erythrocytes they no longer were capable of adsorbing more virus and became refractory to agglutination by virus of either type. The virus removed from the red cell is apparently unaffected and can agglutinate new cells

to undiminished titer. This observation readily led to the concept of specific receptors with which the virus reacted. That the union was not dependent upon the intact cell was shown by the adsorption of virus to the stroma of cells lysed by heating to 65°C. or 100°C. or by mechanical disruption, a fact amply confirmed in our laboratory. Hirst points out the analogy between the inagglutinability of cells from which virus has separated and the effect of an enzyme upon its substrate, in which the two reagents first combine, then the substrate is altered and the enzyme can act upon additional substrate. The agglutinating factor was shown to be more resistant to heat than the infectious activity but was destroyed rapidly at 60°C. Heated PR8 strain was still adsorbed and eluted, but heated Lee virus did not elute. Treatment with formalin removed infectivity but hemagglutinating activity persisted; nevertheless, with 0.1 and 0.5 per cent formalin adsorption and elution were retarded; with larger concentrations adsorption occurred but no elution took place. Hirst commented that the relative sensitivity of the agglutinin might indicate a protein nature while the stability of the cellular component suggests a nonprotein nature.

On the hypothesis that the susceptible tissue cells of the susceptible host contain a similar receptor to which the virus attaches to create the primary cell injury, further studies were made with animal lungs (25). The lungs of ferrets were perfused *in situ* at 37°C., removed, and then suspensions of virus were introduced into the lung through the trachea after which the fluid was removed and titrated for virus activity. It was shown that virus was largely adsorbed by the cells in five to thirty minutes and that after this time virus gradually reaccumulated in the fluid. With the lungs of mice essentially the same results were obtained. The collection of virus in the intrapulmonary fluid was thought to mean that the previously affected cells from which virus eluted, were no longer able to combine with additional virus. Heated or formalinized virus was adsorbed but did not elute from the tissues. Inoculation of virus into living ferrets which were sacrificed at intervals thereafter, to test for virus, indicated rapid adsorption, but virus did not reappear for about twenty-four hours; this could represent multiplied virus. These data furnish additional support for the idea expressed that the union with, and subsequent destruction of, a specific receptor substance, involving an enzy-

matic reaction, may be a necessary preliminary event in the parasitism of susceptible cells by influenza virus.

Burnet and his associates have approached the problem by studying the effect of removal of presumed receptors by one virus or strain upon the behavior of the cells with other viruses. The virus of Newcastle disease behaves as a hemagglutinating agent in much the same manner as influenza virus and it was found that cells which had been treated with Newcastle virus were no longer agglutinable by that virus, though still affected by others (26). Strains of Type A influenza virus removed receptors for themselves and for Newcastle disease virus. Agglutinability of red cells by Type B virus was not affected by previous exposure to Type A virus, a point at variance with Hirst's original observations and our own; nor was agglutination by swine virus disturbed. On the other hand, treatment with Type B virus left the cells agglutinable by only swine virus, and treatment with swine virus removed receptors to all. This graded effect suggested the interpretation that different receptors were of different accessibilities in respect to the surface and were destroyed according to the relative activity of a given virus. Subsequently, the series was expanded to include mumps virus and other strains of influenza virus, Types A and B (27). The extent to which the various strains rendered cells inagglutinable could be placed in an increasing scale. It is of interest to note, however, that there was no clear order of arrangement of strains of influenza virus according to type; it was an individual behavior which might well be dependent upon the strain's experience. Differences were also noted in the arrangement according to whether cells from guinea pigs, fowl or humans were used.

It was found that toxin of *Clostridium welchii*, Type A, or toxin and filtrates of *Vibrio cholerae*, both of which contain lecithinase, also removed the agglutinability of cells to the various virus strains in an order approaching that obtained with the viruses. Evidence that lecithinase was the active principle in this effect was not, however, obtained. Nevertheless, it is a lead of considerable interest in attempting to characterize the nature of the receptors.

Another of the observations was that cells treated by virus were subsequently susceptible to agglutination by a variety of mammalian sera in low dilution. It was then noted (28) that human O cells treated with Newcastle disease virus were agglutinated to low titer by most human sera (>1:160 dil) but that

eight of fifteen sera from patients with infectious mononucleosis agglutinated the cells to high titer. The data are not sufficient to correlate this effect with the heterophile reaction to sheep cells although five of the eight sera with high heterophile titers gave high titers to the virus-treated cells. The authors believe that the cells are antigenically modified rather than merely coated by virus because the capacity of successive wash fluids of the cells showed progressively decreasing power to prepare other cells although actively agglutinating virus was present. The preparing component has not been separated from the virus itself and the other evidence is insufficient to permit a conclusion. It may be that the reaction with sera from infectious mononucleosis is unrelated to the character of the virus employed since no data are presented as to the specificity of reaction. The fact that cells treated in the same way are readily agglutinated by serum against the Newcastle virus indicates in that instance, however, an important part of the reaction is the quantity of adsorbed virus present.

This conclusion is heightened by the further important observation that human O cells treated with mumps virus are agglutinated by serum from mumps convalescents or from individuals inoculated with inactive mumps virus (29). Deposition of the virus antigen at the surface seems to be the important factor, although Burnet suggests the possibility that in both this case and that with Newcastle virus, partially disintegrated virus may be the material which combines with red cells without agglutinating them.

Agglutination of erythrocytes of various species by viruses of vaccinia (30) or ectromelia (31) seems to be of a different order. This action is not tied to the infectious virus unit; it occurs with cells of one or two species and then in a limited manner; it is not eluted from the red cells. The similarity of their action to that of lipids (32) from animal tissues and their inactivation by *Cl. welchii* toxin and cobra venom (33) suggests a lipid nature.

Agglutination of still a different order was first reported by Mills & Dochez (34) with pneumonia virus of mice. It was found that the exuding fluid from the lungs of mice caused agglutination of the animal's erythrocytes while suspensions of the lungs did not. Extracts heated to 75°–80°C. did, however, give excellent agglutination. The apparent release of agglutinating ability by

heat and its inactivation by normal lung tissue or erythrocytes of the mouse indicated that the activity was masked by union with the host's cells. The agent could in either condition immunize and induce antibodies which specifically inactivated it. The agglutinating activity appears to be an integral part of the virus (35, 36, 37).

The fact that the hemagglutinating and infectious activities of influenza viruses are closely associated has been confirmed by numerous workers under a variety of conditions. Hirst's original observation (13) was that a temperature of 55°C. for five to fifteen minutes was capable of eliminating infectivity in allantoic preparations of PR8 and Lee strains while the major agglutinating action was retained; at 60°C. both were removed. Miller (38) noted that the maximum stability of both in phosphate buffer was maintained between pH 6.5 and pH 7.0 with Type A, between 7.0 and 8.0 for swine virus while with Lee, Type B strain, it was higher. At the higher ranges infectivity decreased more rapidly than agglutinating action. Inactivation of the agglutinin of PR8 strain in egg fluid by a temperature of 55°C. was found by Lauffer & Scott (39) to be least rapid at pH 8.4. Salk (40) has, however, shown that the hemagglutinating factors of different strains, or of lines of the same strain, vary in their susceptibility to heat or to formalin and heat at 61°C. which regularly destroys infectivity. In fact, the stability of hemagglutination was enhanced by formalin (41). Jones has reported the adaptation of a line of virus to withstand temperature normally inactivating the original strain (42). Moreover, it has been observed (43) that with new strains of Type B virus maintained in allantoic fluid, heating at 56°C. for fifteen to thirty minutes did not ordinarily reduce agglutinating activity, although infectiousness is destroyed. Some factor was eliminated, however, for no longer did the heated preparations combine readily with specific antibody in serological tests and the action of a nonspecific inhibitor of virus-agglutination present in serum was enhanced. This showed that heating had caused dissociation of agglutination and an antibody-combining factor which may be the infectious moiety.

The removal of agglutinating and infecting activity by heat or other agencies does not necessarily eliminate antigenicity for it has been observed that preparations so treated may in man still call forth antibodies to the virus (44). It seems quite likely, however, that there is a quantitative gradient of destruction.

Toxicity.—Influenza virus also possesses a toxic factor which has been notably observed in the vaccination of human individuals. That it is the virus which gives rise to inflammation at the site of inoculation and the malaise, aching and fever which occur in a proportion of individuals was early indicated by the enhancement of effect when the amount of virus inoculated was increased. Studies with centrifuged material have clearly shown the same result (45). The toxic effect noted in man is not eliminated readily by procedures which inactivate the virus.

Over a period of years the fact that relatively large amounts of virus given to mice or eggs often produced rapid death has suggested the same idea. The experimental studies of Henle & Henle (46, 47) have shown that the toxic principle as measured in animals is intimately related to the virus body and its infectious properties and all efforts to separate them were unsuccessful. With heat, formalin or irradiation the reduction in toxicity parallels the loss of infectious titer, although the latter proceeded more rapidly. The data are not given, but from the protocols it appears probable that the hemagglutinin persists while toxicity and infectiousness are lost. In cultivation of virus in eggs agglutinating titer reaches its peak before toxicity does and with prolonged incubation of the eggs, toxicity is lost while hemagglutination persisted. Type specificity was retained in that toxicity of Type A and Type B strains was best neutralized by the specific sera. There was, however, some crossing of effect with low dilutions of heterologous serum and of normal rabbit serum. Differences in toxicity of strains was observed. Several of these points were confirmed in the studies by Hale & McKee (48).

Intracerebral inoculation caused destruction of ependymal cells and fatal convulsive seizures in one to three days (46, 48). Intravenous or intraperitoneal injections caused death in eight to ninety-six hours with widespread necrosis of liver and spleen, intestinal hemorrhage, and pleural effusion. These lesions were shown not to be due to multiplication of virus. Evans & Rickard (49) demonstrated that inoculation of virus into the anterior chamber of the rabbit's eye produced corneal opacities of a toxic nature which were closely tied to virus activity and could be specifically neutralized.

The marked toxic manifestations of clinical influenza are out of proportion to the pathological features in the usual case of the disease. It is of interest then that the virus particle should possess

a toxic principle intimately associated with its infectious property similar in character to that described by Rake and Jones for the psittacosis-lymphogranuloma group (50). To what extent the factor studied in animals is related to symptomatology or to the effect of inoculations in man is not clear but additional studies along this line are warranted. It has been suggested that the toxic factor may be a significant participant in the measurement of antibody to hemagglutinating factor and that the nonspecific inhibition of virus agglutination by normal serum may be related to an antitoxic activity of the animal serum acquired from heterogenetic sources (43).

Complement-fixing antigen.—In 1937, Hoyle & Fairbrother (51) stated that the complement-fixing antigen of influenza virus was not sedimented from preparations of infected mouse lung and took this to indicate that the complement-fixing substance was a soluble antigen separate from the bodies causing infection. Lennette & Horsfall (52), however, were unable to obtain infectious virus free from complement-fixing antigen and in explanation called attention to the low potency of the material Hoyle and Fairbrother used. They did, nevertheless, report that after centrifugation to remove the greatest portion of the infectious moiety a considerable portion of complement-fixing antigen remained in the supernatant. Filtration studies suggested that the complement-fixing fraction passed filters considerably tighter than those which ordinarily retain infectious virus but comparative titrations were not done. The material had characteristics of protein. Henle *et al.* (53), using allantoic fluid, reported that centrifugation at 90,000 g. (30,000 r.p.m.) for sixty to ninety minutes concentrated the great proportion of agglutinating activity in the sediment although the complement-fixing titer was higher in the supernatant fluid. In contrast, the data show one instance in which the original fluid collected after eighteen hours' incubation and the supernatant after centrifugation showed no complement-fixing antigen while the sediment did. Furthermore, in other tests for optimal proportions, there was a sharp reduction of agglutinating and complement-fixing titers of the supernatant; the reconstituted sediment reacted with the same or higher dilutions of serum than did the original fluid; the effective dilution of sediment was half that of the original, a modification which might well be ascribable to aggregation. A similar effect was obtained in preparations resulting

from one absorption of allantoic fluid with adult chicken's erythrocytes but a second absorption with erythrocytes removed further activity from the supernatant. When, however, centrifuged supernatant and rediluted sediment were treated with erythrocytes the complement-fixing antigen was largely adsorbed from the sediment, while the activity of the supernatant was essentially unchanged. Absorption of convalescent human serum with sediment removed complement-fixing antibody so that no reactions occurred with the original fluid, the supernatant or the sediment. Absorption of serum with nonsedimentable material, obtained through ammonium sulphate precipitation and dialysis, removed a good portion of the reaction of serum with the original fluid or sediment, and all reaction to the supernatant was eliminated. The serum-absorption experiment was interpreted to mean that the major and more active part of the participating antigen in the sediment was similar to the nonsedimentable fraction. Washing of sediment as many as four times did not change its titer although antigen presumed to be small component was given off into the wash fluids. In fact, the authors state that the specific antigen of sedimentable material constituted a relatively small proportion of the total antigenic activity of the sedimentable material, but it is difficult to see this evidence in the data. The authors seem to put great emphasis on the experiment in which, when sediment and supernatant were mixed, the same optimal titers of serum and antigen were observed as with the original fluid; the resuspended sediment reacted with a higher dilution of serum than the original fluid did but could not be diluted to the same extent as the original. Antigenic activity of the supernatant was definitely reduced, but present. They insist an additive effect should be obtained but the differences are of no significance when the possible aggregative effects related to centrifugation are considered. The investigators point out that the infectious activity of virus in the egg declines after forty-eight hours and that the sedimentable component adsorbable by red cells decreases; the nonsedimentable, nonadsorbable fraction increases although the agglutinating and complement-fixing titers remain about the same. This of itself strongly suggests that the nonsedimentable portion is made up of degradation products of the complete virus, which may be still reactive in the complement-fixation test with certain sera but do not combine with erythrocytes. Certainly, the data seem in keeping

with the idea that the latter is an incomplete fraction of the sedimentable reagent and the conditions of preparation of the fluids for most experiments would favor its presence. The eggs had been incubated for ten days prior to infection; residual antigen in the supernatant was definitely greater in eggs incubated for more than forty-eight hours after infection than with shorter periods of incubation. As will be seen (60), that corresponds with the period in which a normal component rapidly increases in the egg. The fact that multiple human sera were employed could also introduce variables. Moreover, the fact that in most instances the supernatant fluids still contained agglutinating and infectious virus offers obstacles in interpretation. It has not been clearly shown that certain of the effects may not be due to physical changes resulting from centrifugation, yielding larger aggregates in the sediment and ruptured virus in the supernatant furnishing more finely dispersed particles which would react more readily with antibody.

Friedewald's results (54) obtained with allantoic fluid from eggs incubated forty-eight hours after infection and strain specific convalescent ferret sera, were in general agreement with the foregoing. He noted that much more of the nonsedimentable antigen occurred in preparations from mouse lungs than in allantoic fluid. There was a sharp strain specificity with sedimented antigen and less with the residual. The PR8 strain appeared to possess much less of the "soluble" antigen than did the WS. The sedimentable antigen was not separable from the virus particle; it sedimented and adsorbed and eluted from red cells with hemagglutinin. The serum titers with "soluble" antigen were considerably lower than with the other and less strain specific. Later, Friedewald & Pickels (55), by centrifugation in a sucrose density gradient so as to reduce convection, noted differences between PR8 and Lee strains. The former sedimented with a diffuse boundary yielding particles of 700 S associated with infectivity and hemagglutination; the 460 S component was still adsorbed by red cells but had a limited infectiousness. The Lee strain showed essentially a single boundary of monodisperse particles of 800 S. But in both, a small component making up 5 to 10 per cent of the material appeared to be associated with complement-fixation but not with infection. Its varied rate of sedimentation was taken to mean various stages of disintegration of the virus particles. Hence, they conclude that most of

the C-F antigen is intimately associated with the primary infective virus particles.

Hoyle (56), however, contends that if this "soluble" antigen were a disintegration product several different antigens should be found since the virus particle is made up of a number of antigens. Using mouse virus suspensions of different but related strains he noted that the optimal concentration of soluble antigen from one strain was the same with four different human convalescent sera and that a given serum had the same maximal titer with soluble antigens of different strains. The data are interpreted to indicate that the soluble antigen is common to all strains of the type while the sedimented one shows strain specificity. The evidence does not, however, appear conclusive since the behavior of disintegrated particles cannot be prophesied in terms of the constitution of an intact material. The concentration of reconstituted, sedimented antigen for optimal reaction was only one-quarter as great as that for the soluble portion and less serum was required; this indicates a greater antigenic activity in sedimented material in contrast to the interpretation of Henle *et al.* (53)

Friedewald (57) in turn states that from specific convalescent serum of ferrets the complement-fixing, the neutralizing and agglutination-inhibiting antibodies are completely absorbed only by the homologous strain. Strains exhibited different capacities to remove heterologous antibody; PR 8 strain was the most effective. These results are not completely in accord with Hoyle's interpretations for if a soluble antigen from all Type A strains is the same, proper preparations of different heterologous strains should absorb homologous antibody to the same extent. Of course, this could be attributed again to quantitative differences in output of soluble antigen by different strains.

Henle & Wiener (58) in further studies with more purified allantoic fluid preparations observed three fractions called 600 S, 30 S, and <30 S, according to their sedimentability. The latter two behave the same serologically while the active virus antigen was considered on the basis of serum absorption tests to be another antigen. The small antigen from either of two Type A strains absorbed all antibody reacting with the small component; the large antigen from homologous strain removed antibody to both the homologous and heterologous 600 S but the heterologous strain

did not completely remove antibodies to the homologous 600 S. The small component is now considered to be released from the virus with its disintegration; support for this idea was found in the fact that high speed oscillations apparently released a similar fraction from the large antigen.

Wiener, Henle & Henle (59) have recently reported that only 15 to 20 per cent of individuals receiving vaccines of influenza virus responds with CF antibodies to the 30 S fraction while the majority does after infection, suggesting the application of the technic to that troublesome problem of serological diagnosis of influenza in vaccinated individuals.

In the studies concerning the nature of complement-fixing antigens it becomes apparent that in order to reach a point of clarification there is a great need for a certain uniformity in procedure. Strains of similar characteristics, maintained under similar conditions in eggs or mice and employed in similar serological systems should be employed. Consideration of the proper controls of the mechanical and biological changes which may take place in the handling is also needed. Further points of interest stand out in the studies of the small component in normal tissue products.

Normal tissue component.—The nature of the small component is considered in the studies of Knight (60) who isolated from normal allantoic fluid material having certain characteristics of the small component already discussed. It is found in ultracentrifuged concentrates of virus, moves electrophoretically like the 30 S component, sediments in an irregular manner, has an estimated size of 40 mμ, is not adsorbed by red cells, has a chemical composition similar to that reported for influenza virus. The material rapidly increases in fluid of embryos after the twelfth day of incubation. Moreover, presumably purified preparations of influenza virus obtained by centrifugation or by adsorption and elution from red cells reacts with serum to the normal protein. The amount present in fluids infected with virus is considerably greater than in normal fluid and in preparations of virus concentrated by centrifugation constitutes from 30 to 50 per cent of the presumed virus. Absorption of specific rabbit antisera with centrifuged PR8 strain of virus removed precipitins for the normal protein and the virus whereas, absorption of PR8 serum with the normal protein removed only precipitins to the protein and strong reactions to the virus remained.

In later studies Knight (22, 61) worked with virus prepared from allantoic fluid and mouse lung. Either PR8, Type A, or Lee, Type B, centrifuged from allantoic fluid precipitated serum against normal allantoic protein while preparations from mice did not. However, serum against PR8 virus from allantoic fluid precipitated with normal allantoic protein and with the PR8 virus from either allantoic fluid or mouse lung. Normal mouse tissue did not react.

The antibody removed by Lee virus had no antiviral influence against PR8 while that removed by the PR8 contained specific neutralizing and agglutination-inhibiting activity. Serum against normal mouse lung precipitated with normal mouse lung and with virus from mouse but not with that from allantoic fluid or with normal allantoic protein. The capacity of mouse lung virus to react with antimouse serum was lost after one passage in eggs. Knight states that since adsorption of virus on red cells does not result in adsorption of the normal component, as was earlier shown by Taylor *et al.* (21), but specifically absorbs only virus, the presence of normal tissue component in such material is strong evidence for its being a constituent of the virus. The objections to this interpretation are obvious. Anti-viral serum reacts with viral activity regardless of its origin and absorption of serum with normal constituent does not remove antibodies to the virus although it might be expected if the host component constituted part of the virus, especially since the biological activity of allantoic virus is precipitated by serum against normal allantoic protein. Moreover, the reaction described is the only serological one in which the sharp type distinction between Type A and Type B strains has been obliterated by a common response. In this respect the normal component differs from the small component of Henle, which was type specific, although it would be interesting to know how that substance would react with serum to the normal allantoic component. Despite the fact that the evidence presented by Knight scarcely seems to justify the conclusion that the host fraction is an integral part of the virus, the idea that it could be the case is not surprising, nor is it at all new.

In view of the different origins of the materials and the presence of presumably different host proteins it is of interest that Knight's chemical analyses of virus from either egg or mouse material were generally similar and that they agree in general with those com-

TABLE I

Chemical Analyses of Influenza Virus

Author	Virus or normal noninfected fluid	Carbohydrate as glucose	Alcohol-ether sol. fract.	Carbon	Hydrogen	Nitrogen	Phosphorus	Nucleic acid	Rib. nucleic acid	Ribose	Desox. ribo nucleic acid	Comments
A. R. Taylor, *J. Biol. Chem.*, 153, 675–686 (1944)	*Allan. fluid*											
	PR8	12.5	32	53		10	.97				2.1	A portion of the carbohydrate is probably mannose+glucose-galactose and is not associated with nucleic acid
	Lee	13.1	30	53		9.7	.94				3.7	
	Swine	10.0	30	51		9.0	.87				+	
C. A. Knight, *J. Exptl. Med.*, 80, 83–100, (1944)	*Allan. fluid*											
	PR8	6	27	52	7.7	10	1.0	5	+		+	Little difference in chemical determinations of infected and normal allantoic fluid sediment. Implication of a complex of normal protein virus.
	Norm Fluid Component	6	22	52	7.7	10	1.0	5	+		+	
C. A. Knight, *J. Exptl. Med.*, 83, 11–24 (1946)	*Mouse lung*											
	PR8	6.2	38			10.1	1.06		+		+	More marked differences in infected and normal lungs than in infected and normal allantoic fluid.
	Norm. lungs	3.5				6.9	1.40		+		?	
C. A. Knight, *J. Exptl. Med.*, 85, 99–116, (1947)	*Allan. Fluid*											
	PR8	6				10	1.0	5	2.3	.7	T	4 to 5 per cent nonnucleic acid carbohydrate. The carbohydrate seemed to be a polysaccharide comprised of mannose, galactose and glucosamine.

(All values are listed as per cent of total virus or sedimented complex.)

piled by Taylor. Cohen points out that the validity of chemical results as characterizing the virus is dubious (62). Furthermore, if the tissue component constitutes as large a portion of the supposed virus material as is suggested its effect upon physical measurements must be taken into account.

Chemical determinations.—Taylor has summarized the analyses of influenza viruses which he and his associates have made. The accompanying table includes their data and those of Knight (Table I).

Serological variation.—The significance of serological variation in strains has continued a challenging problem. With large scale studies of prophylactic vaccination the antigenic spread obtained with a given strain is of importance if the sharpness of serological distinction is actually a reflection of immunological independence. It is a common experience for strains newly isolated and propagated in eggs to appear antigenically remote from established strains (2); some of this effect is related to the enhanced agglutinin-inhibiting effect of normal serum of all sorts so as to obscure the measurement of specific antibody. It has been repeatedly observed, however, that the serological behavior of egg passage strains may be different from the same strain in mice. Stuart-Harris (63) has pointed out some of the inconstancies encountered. Different results are also obtained in tests using immune sera from different species. For example, strains from the 1945 epidemic of influenza B appeared with rabbit sera to be almost unrelated to standard Type B strains; with ferret sera they seemed to be sharply different from the standard Lee strain but with human sera they behaved as characteristic B strains. It is obvious that the specificities of antibody encountered in the animal and human sera are different, and this fact must also be considered in terms of antigenic stimulation produced by vaccination. Nevertheless, the strain differences exist and are potentially great obstacles to effective immunity. Strains with broadest distribution of antigenic constituents are to be sought for the purpose of overriding specificity. (A Strain Study Center for these purposes has been established by Dr. T. P. Magill for the Commission on Influenza, Army Epidemiological Board). In this respect the PR8 strain has always been among the most diffusely antigenic and for that reason has appeared a desirable strain for immunizing purposes. In the investigation of the effect

of vaccination carried out by the Commission on Influenza in 1943, Eaton & Meiklejohn (64) have proposed that the reason for their indefinite results as compared with the other studies was related to the fact that the vaccine, containing the PR8 and Weiss strains of A, had not been so efficient in eliciting antibodies to the strain in epidemic circulation in that area as to the vaccine strains; there were, however, other deviations as well. In the Michigan study (65) there was evidence suggesting that the epidemic strain was perhaps more closely related to one vaccine strain than to the other. Quantitative differences of these degrees, however, are difficult to assess and Hirst (66) has pointed out that different animals receiving the same agent may respond differently as regards the sera they produce and that different strains which appear to be widely different can be shown to be closely the same when results are corrected in this light. Moreover, the strains of Type B virus encountered in 1945–46 (2) appeared to be quite different from the Lee strain in the vaccine under certain conditions of testing. Vaccination had, however, resulted in antibody rises to those strains and apparently protected against disease caused by them. In England (67) it was also observed that vaccination elicited the same quantitative output of antibodies against strains which appeared antigenically different from Lee strain as against the Lee itself; in contrast the natural infection in some instances gave more antibody to the current strain than to Lee but great variation was observed, showing that the response of previously experienced hosts is not uniform.

It is not possible, however, to state how widely divergent a strain can be and be encompassed effectively by the response to another strain of the same type. The swine strains of Shope can be shown to be related to Type A strains and antibodies developing after influenza A infection, even in infants (68), neutralize swine virus but ordinary procedures of immunization with A viruses do not protect against significant amounts of swine virus. The wide range of serological response is clearly indicated.

One other form of variation is of interest in this connection, the O-D change of Burnet & Bull (69). In the isolation by amniotic route of strains of Type A influenza virus from throat washings of patients they noted that the amniotic fluid containing virus gave a low titer of agglutination with chicken erythrocytes but much higher titers with guinea pig cells. On successive passages this behavior changed so that agglutination of chicken cells was as high

or higher than that of guinea pig cells. On the other hand, if high dilutions of amniotic fluid were used for transfer it was possible to maintain a high guinea pig-fowl ratio, presumably by selection of virus particles in the proper phase through dilution (70); transfer of stronger dilutions of the same material produced virus which acted well with fowl cells. These authors propose the hypothesis that the original O behavior of the virus in agglutinating guinea pig cells better than chicken cells represents the natural state in which virus exists in the human while the D phase derived by passage in which chicken cell agglutination is prominent is a mutation from the human type. The O form does not multiply in the allantoic cavity; D does. D infects mice more readily and grows more rapidly in the chick embryo; it is presumed to lose its capacity to infect man by transfer. The O form can immunize animals, though not adapted to them, but apparently less effectively than D. The observations are of interest although the evidence to support the extended thesis is incomplete. In 1943, Rickard, Thigpen & Crowley (18) recovered virus from nine of sixteen throat washings inoculated into the allantoic sac; in our laboratory eight of eighteen were positive, some with gross agglutination of chicken cells on first passage, and no evidence was obtained of higher agglutination of guinea pig cells on first passage (65). Burnet, Stone & Anderson (71) state that all the Type A strains they isolated in 1945 showed the O state on primary isolation; they had earlier, however, found D form in some cases. They failed to note O-form in B virus isolated in the amniotic sac; in our laboratory, in 1945–46, repeated isolations of Type B were made by allantoic inoculation even on first passage, but more amniotically (2). The new strains agglutinated chicken cells to good titer. There is evidence that the amniotic route may yield a greater number of isolations directly from the patients' throats than the allantoic but this is to some extent, at least, related to factors other than O-D effects. The frequency of the O phenomenon can apparently vary with epidemics; hence, it is not established that D forms are less capable of transferring disease in man.

Nevertheless, the use of an index such as described for O forms may be of considerable value in helping to identify an agent in a state of activity which is ordinarily difficult to detect or to stabilize. In certain protocols the data suggest that the O reaction is most obvious when virus is low. It apparently is not related to the

agglutination observed with amniotic fluid inoculated with non-infectious material (72). But that only the O form represents the usual human pathogen cannot be presently accepted as established.

Psittacosis—Lymphogranuloma-venereum Group

One series of infectious agents commonly classified as viruses has been increasing in number and in biological significance. The group originally formed about the viruses of psittacosis, lymphogranuloma-venereum and meningopneumonitis, now includes viruses of trachoma and inclusion blennorrhea, of ornithosis, of pneumonitis of mice, of man, of hamsters, of cats. The remarkable feature of this family is the widespread distribution in various species of fowl and animals and the probability exists that its range will be further extended as studies in other species progress. In most instances the agent apparently exists as a resident or carrier infection which becomes activated by physiological disturbances, as with the parrot which is chilled or shipped; or the ferret or mouse which is inoculated with material in a search for other viruses. Moreover, a good percentage of apparently recovered animals tends to remain carriers of the different agents and thus furnish the means for subsequent relapses. It is clear from reported observations with birds (73) and mice (74) that infection can be incurred without overt evidence of it and there is clear invitation to extend the concept that in the respective native species the various agents, including lymphogranuloma venereum, can be looked on as primarily saprophytic parasites. In view of the general behavior of the group as a whole one might well anticipate the recognition of a respiratory form of latent virus in man, as suggested by Smadel (75). The apparent recovery of meningopneumonitis virus from men, without relation to their disease, would support this concept (76).

The agents are characterized by large cytoplasmic inclusions, breaking into elementary bodies, which can be found outside the cells as well as within, in a cycle which has been much the same in the cases studied as that first described by Bedson & Bland for psittacosis (77), Thygeson in trachoma (78), Thygeson & Mengert in blennorrhea (79), Rake & Jones in lymphogranuloma venereum (80).

Rake, Eaton & Shaffer (81) emphasize the morphological similarities of the viruses of psittacosis, lymphogranuloma-venereum

and meningopneumonitis, and clearly showed that an antigen reacting in the complement-fixation test was common to all. These results have extended through the group although in one series the serum of pigeons, naturally infected with ornithosis but apparently well, gave little or no reaction with lymphogranuloma antigen (82). It has been observed otherwise that variations in antibody reaction to heterologous and homologous strains are prominent. Various procedures have been employed in efforts to establish similarities and individualities among the agents.

The pathogenicity of these viruses for different species of animals and by different routes has yielded information (83, 84) tending to show two groups according to intracerebral effect—the mouse pneumonitis viruses which have not caused fatal disease after intracerebral inoculation of mice; the others do. Psittacosis, ornithosis and meningopneumonitis can cause fatal infection of mice by intraperitoneal inoculation; psittacosis does not take intracerebrally in pigeons while the other two do; lymphogranuloma and the S-F strain of human pneumonitis do not produce fatal disease or carrier state after intraperitoneal but do after intracerebral injection. The lack of pathogenicity for birds sharply differentiates the viruses of lymphogranuloma-venereum and mouse pneumonitis from the others. But some of the variations noted in this type of testing seem scarcely sufficiently sharp to be conclusive, particularly since differences from one strain to another may prove with further study to be equally as great. Moreover, some of the differences are clearly quantitative, such as whether infection is fatal or not, or whether doses of quite disparate size must be used to obtain a similar effect.

Cross immunity tests in mice have shown that meningopneumonitis and ornithosis viruses are essentially the same. They also appear to be the most effective in inducing immunity to intracerebral test against heterologous psittacosis and S.F. strains, while the latter is a poor antigen except against itself (83). Here again the differences appear likely to have a quantitative character since results obtained by intraperitoneal testing showed cross immunity (85). The mouse pneumonitis strains give cross immunity to one another (84).

Neutralizing antibodies have in most instances been difficult of significant demonstration but Hilleman (86) and St. John & Gordon (87) prepared sera of chickens immunized with different viruses.

In the former study sera were prepared against M-P, LGV and mouse viruses and neutralization tests were done intranasally. In the latter, sera against Ann Arbor mouse virus and feline pneumonitis virus were added. Here again, the viruses of meningopneumonitis and ornithosis cross-neutralized as did the various strains of mouse pneumonitis. The other viruses tested appeared to be specific.

Rake & Jones (50) have also demonstrated a specificity of strains by neutralization of toxin. This principle appears to be an integral part of the virus, inseparable from it, and resembles bacterial endotoxins in its action. Intravenous inoculation of mice produces death in four to thirty-six hours with marked midzonal hepatic necrosis, splenic necrosis, and pulmonary edema and hemorrhage. Toxins prepared in yolk sacs with viruses of lymphogranuloma-venereum, meningopneumonitis, mouse pneumonitis or feline pneumonitis (88) were specifically neutralized by homologous serum as shown by elimination of the early fatalities but the sera did not (except with lymphogranuloma virus) prevent the subsequent deaths from infection. Moreover, sulfonamides were found to influence infection of mice but not the early death from toxin (89). The phenomenon, as that with influenza virus, opens a different approach to identification and study of these agents.

The second most interesting characteristic of this group of viruses is that they represent the only ones which have been found susceptible to sulfonamides and penicillin. Experimental and clinical studies with sulfonamides began early to show promising results with lymphogranuloma-venereum, trachoma and inclusion blennorrhea. This subject has been well summarized by Jones, Rake & Stearns (90). The virus of mouse pneumonitis was shown by Rake, Jones & Nigg (91) to be susceptible to treatment. Parker & Diefendorf (92) noted that penicillin retarded the growth and effect of psittacosis and meningo-pneumonitis virus in chick embryos while Heilman & Herrell (93, 94) observed the advantageous effect of penicillin in infection of mice.

A summary of experimental therapeutic effects with penicillin and sulfadiazine against a series of these agents in mice and chick embryos has recently been given (95, 96) (Table II).

It is engaging to note that penicillin had measurable effect against all strains tested although the susceptibility of the psittacosis group was less than that of the other members. On the other hand, two typical psittacosis strains (6 BC and Gleason)

differed widely in their sensitivity to penicillin. The meningopneumonitis, ornithosis and closely allied human viruses were unaffected by sulfadiazine. Eaton & Hanford (97) have observed more definite effects on virus of feline pneumonitis treated with large doses of sulfamerazine; the effect to some extent is related to the host used for testing.

TABLE II

SUSCEPTIBILITY OF STRAINS IN THE PSITTACOSIS-LGV GROUP TO PENICILLIN AND SULFADIAZINE IN CHICK-EMBRYOS AND MICE
[Ref. No. (96)]

Virus	Strain	Penicillin		Sulfadiazine	
		Chick-Embryo	Mouse	Chick-Embryo	Mouse
Psittacosis	6BC	+	+	+	+
Psittacosis	Gleason	+	+	+	+
	Borg	+	+	0	0
	SF	+		0	0(?)*
Pigeon ornithosis	P207	+	+	0	0
Meningopneumonitis	Cal 10	+	+	0	0
Feline pneumonitis	Cat	+	+(?)	0	0
Mouse pneumonitis	Nigg	+	+	+	+
Hamster pneumonitis	12XN	+		+	+**
Lymphogranuloma venereum	LGV	+		+	+***

+ Susceptible.
0 Not susceptible.
* Based on observation that death was not delayed in mice challenged by intracerebral route.
** Eaton, M. D., and Hanford, V. L. (97).
*** Rake, G., Jones, H., and Nigg, C. (91).

To a large extent the effect of drugs has been suppressive as shown by the fact that the agents multiply readily in chick embryos when the drugs are stopped. In mice a considerable proportion of the animals continue to carry virus after apparently effective treatment.

The mechanism which renders this group of viruses susceptible to drug action may lie in part in their more complete structure. In addition, however, the virus particles have a definite extracellular phase in the progression of infection. Nigg and Eaton refer to

phagocytosis of elementary bodies by the polymorphonuclear cells (84). Jones, Rake & Stearns (90) discuss a stage of extracellular metabolism. It seems likely on the basis of present information that the effect of drug is exerted on the stage outside of the large mononuclear cell. Moreover, the response of the host tends to be exudative and proliferative rather than productive of early cell destruction, another feature which may well add to the susceptibility of the infections to treatment. But the fact that this group of agents, viruses in their intracellular habit and in the requirement of living cells for multiplication, is affected strongly, indicates that they may serve as the keystones for study of chemotherapy of virus diseases. The clinical data continue to indicate that the course of psittacosis can be favorably influenced by treatment with penicillin instituted early and continued in large doses (98).

Rake (90) has commented on the question presently discussed of whether these agents should be classified apart from the viruses. There is no doubt that they have features which distinguish them from many of the others. The definition of viruses as a whole is not at present distinct. The psittacosis-lymphogranuloma-venereum group has been placed in a family designated Chlamydozoaceae by Moshkovsky (99) and this classification is being adopted in the new edition of Bergey's Manual. There are historical reasons for disagreement with the generic terms and they may subsequently have to be changed. The proper biological position of these agents cannot be finally settled at present; they bear resemblances to the rickettsiae on the one hand and to the pox viruses on the other hand, suggesting that the latter may well be the next group of viruses to be found susceptible to drug action.

Atypical Pneumonia

With the increased consciousness of the rôle of viruses in respiratory disease there evolved a realization that a form of pneumonia was prevalent which did not conform clinically or bacteriologically to the common bacterial pneumonias. Moreover, it failed to respond to therapeutic doses of sulfonamides. These factors combined to elicit the suggestion that the disease was caused by a virus and the term "virus pneumonia" was applied. The various episodes reported clinically were not, however, of sufficient uniformity to indicate that the same condition was being described. As military mobilization proceeded in 1940 an enhanced incidence of atypical

pneumonia became apparent and the pattern of the disease, which has been thoroughly reported by many observers, was such as to approach the probability of an etiological entity.

Efforts to establish an etiological virus have yielded multiple reports. The first evidence of an agent transmissible to animals was reported by Reimann (100) on the basis of evidence obtained by Francis & Magill (100), and by Stokes, Kenney & Shaw (101). Material from the same cases was studied in both laboratories, resulting in evidence that mice and ferrets were affected by intranasal or intracerebral inoculation of throat washings or blood. The agent was not definitely established and its nature was not determined; nor was its relation to the disease demonstrated.

Weir & Horsfall (102) reported the establishment of a virus in the mongoose after intranasal inoculation of sputum or throat washings of patients with acute pneumonitis. The agent was filterable through Berkefeld V and N filters; it was maintained by passage in chick embryos; its capacity to produce pulmonary lesions was reduced by serum of convalescent mongooses or by that of convalescent human patients; normal mongooses in contact with infected ones also developed pulmonary lesions. The work was not continued and efforts to repeat it by other investigators were unsuccessful (103). Baker (104) recovered a virus from cats suffering from pneumonia; on the basis of preliminary serological tests he suggested a relationship of the agent to human atypical pneumonia. It was subsequently shown, however (105, 106), that the agent was a member of the Chlamydozoaceae and that there was no significant relationship to the usual human disease. Blake, Howard & Tatlock (107) demonstrated a virus from sick cats belonging to a family in which four cases of atypical pneumonia occurred. The agent produced pneumonitis and was serially transmitted in cats and kittens but not in mice. The agent was filterable through an L3 Chamberland filter. Further evidence of its possible relation to widespread disease of man has not been reported. Rose & Molloy (108) described the production of pulmonary lesions in newly weaned guinea pigs by the intranasal inoculation of sputum of patients with atypical pneumonia. The filterable agent was transferable to cotton rats with guinea pig material but not directly with human material. Serological tests with serum of patients failed to demonstrate any relationship.

Horsfall *et al.* (109) reported that the intranasal inoculation of

sputum from atypical pneumonia would produce lesions in the lungs of cotton rats. Animals so inoculated developed antibodies to a pneumonia virus of mice (PVM) as shown by neutralization tests with that virus. The serum of the animals after inoculation and of certain patients recovering from atypical pneumonia also appeared to inhibit the capacity of sputum to produce lesions in the lungs of cotton rats. Material from serial passages in embryonated eggs which had been inoculated with sputum appeared to contain the agent since its injection into animals seemed to elicit antibodies to PVM. The data were interpreted to mean that the causative agent of atypical pneumonia in man was antigenically similar to PVM and that the antibodies so measured resulted from a common antigenic constituent. Subsequently they (110) noted that cotton rats and hamsters developed the same kind of pulmonary lesions after intranasal inoculation of normal materials and that cotton rats, rabbits and hamsters commonly developed antibodies to PVM after these inoculations. It was demonstrated that a virus of this nature was latently resident in the various species of animals. The lesions and the antibodies resulting from inoculation were then attributed to activation of virus latent in the experimental animals rather than to an agent in the sputum of human patients.

Eaton, *et al.* (111) had earlier described the production of lesions in the lungs of cotton rats with some samples of sputum, throat washings, or lungs from patients with atypical pneumonia. It seemed to be especially effective when the specimens were obtained early in the disease. Recovered animals were resistant to reinoculation. With passage some strains were maintained in cotton rats and hamsters and the sera of recovered animals, also those of rabbits repeatedly inoculated, were said to neutralize the agent in tests made in cotton rats and hamsters. In subsequent papers (112, 113, 114) the authors report that material from human sources filtered through membranes of 600 mμ a.p.d., infected chick embryos by amniotic inoculation as shown by the capacity of suspensions of embryonic tissues to induce pulmonary lesions in hamsters and cotton rats. Embryos of eleven days' growth were used and incubated five days after inoculation. Neutralization tests with sera from patients in the acute and convalescent stages of atypical pneumonia, were done by mixing the suspensions of inoculated embryos with the respective sera and inoculating groups of cotton

rats or hamsters intranasally with the mixtures. The investigators report consistent differences; in one series from eight patients inoculation of embryonic material and acute phase sera resulted in lesions in nineteen of thirty cotton rats; the convalescent serum mixtures elicited lesions in one of twenty-nine rats. Some evidence of cross immunity in animals inoculated with human material or with egg passage material was presented. Nevertheless, great difficulty was experienced with contaminating viruses or other agents in the various species of animals employed. It is obvious that PVM virus was circulating since normal rabbits, and rabbits inoculated with human material or chick embryo, exhibited antibodies to PVM. A similar virus in hamsters was commonly present and another designated W2 was repeatedly isolated from cotton rats. Efforts were made to determine their significance in the results by rather limited tests of cross immunity after intranasal inoculations but two of the four agents encountered were not included in the tests. However, hamsters given intraperitoneal inoculations of a known strain of hamster pneumonia virus were found to be immune to intranasal inoculation with that virus but still developed the anticipated lesions when embryonic passage of the presumed human agent was given intranasally. The size of the agent was estimated by filtration to be 180–250 mμ. Examination of additional sera taken at different stages of the human disease resulted in a substantiation of earlier tests; there was in convalescent serum a neutralizing effect upon the lesion-producing capacity of egg passage agent which was ordinarily absent or present in only low titer in the serum obtained during the acute illness. The increases in titer were from four, to sixty-fourfold in 42 of 69 cases of atypical pneumonia. Although certain pertinent data are not included in the tables, titrations of these sera against viruses of mouse or hamster pneumonia ordinarily showed no significant changes, nor was neutralization of two of the other contaminating viruses observed. Increase in effect was not obtained with convalescent serum from other forms of pneumonia and acute upper respiratory infection. Neutralization tests in chick embryos were inconsistent.

It is impossible to assay the presumed virus agent involved in these studies; the specter of contaminating viruses is prominent nor do the experimental data eliminate it. Certain efforts to answer that criticism were made but the results presented are inade-

quate. Nevertheless, the results of neutralization tests with acute and convalescent sera tend to follow a consistent, reproducible pattern. Consequently, they invite consideration as to their specificity and as to their possible relationship to the streptococcal agglutinins and hemagglutinins encountered in atypical pneumonia.

The only direct evidence of a virus in the causation of atypical pneumonia is that derived from studies in human subjects. Vance, Scott & Mason (115) failed to transmit infection in seven individuals inoculated intranasally with 20 to 40 cc. of Berkefeld V and N filtrates of nasal washings and sputum from atypical pneumonia patients. Nevertheless, the Commission on Acute Respiratory Diseases carried out a series of studies with positive results in human subjects. The first experiment (116, 117) comprised twelve men who were sprayed with a pool of untreated sputum and throat washings from cases of atypical pneumonia. Ten of the twelve developed evidence of respiratory infection seven to twenty-two days later. Eight had fever; five of them presented auscultatory evidence of pulmonary involvement, three with minimal roentgenographic changes. Two had mild afebrile disturbances. Because three of the fifteen originally selected developed respiratory disease in a five-day period of observation prior to the date selected for inoculation and were then excluded, there remained a possibility that the infections observed in the test subjects were spontaneous. The second experiment (117, 118) involved thirty-six men who were divided into three groups of twelve each and sprayed, respectively, with untreated sputum and throat washings, with the material filtered through sintered glass or Seitz pads, or with the same material autoclaved. The men had been isolated in individual rooms three weeks before inoculation and throughout the subsequent period of observation. The material was sprayed or instilled into the respiratory tract of the individuals at three intervals in the course of a day, with a separate room used for each group. The results are seen in the accompanying table (Table III).

Thus, atypical pneumonia developed after eight to fourteen days in ten of the individuals, including three who had received autoclaved material. In addition two of the staff who participated in the study developed atypical pneumonia eleven and twelve days after the spraying.

The study was repeated (117, 118) with increased precautions,

including that of conducting the spraying out of doors with intervals of several days between the inoculations of the different groups. The material was obtained from six of the patients with atypical pneumonia in the second experiment, hence, it represents a serial passage. In this series, involving forty-two men, three each of the groups of twelve men receiving filtered or untreated inoculum developed atypical pneumonia, and five in each group had minor respiratory illnesses; one of eighteen receiving autoclaved material developed a minor respiratory illness but no other illness

TABLE III

INDUCTION OF ATYPICAL PNEUMONIA IN MAN

No. of Men	Inoculum	Atypical Pneumonia	Minor Illness	No Illness
12	untreated	3	9	0
12	filtered	4	5	3
12	autoclaved	3	1	8
36		10	15	11

occurred. The onset of illness in those receiving unfiltered material was in general a week earlier than in the group inoculated with filtrates.

The results strongly indicate that a filtrable nonbacterial agent is implicated in the infection and, as suggested by earlier epidemiological observations, that nonpneumonic infection is an integral—probably the principal—manifestation of the transmissible disease (105). The authors, however, consider the possibility that two viruses may be involved, one of which gives rise to the upper respiratory illness, the other to atypical pneumonia.

Certain serological phenomena have been encountered in the course of atypical pneumonia which are of extreme interest in their possible application to interpretation of the causation of the disease. The first of these is the appearance of cold agglutinins (119, 120), that is, agglutination of human "O" red cells by the serum of the pneumonic patient when the mixtures of serum and erythrocytes are allowed to stand at 0° to 4° C. When the mixtures are then placed at room or incubation temperature, the agglutination dissociates. The phenomenon has been encountered in a varied per-

centage of cases and, in general, is most commonly present and of highest titer in the more severe cases of illness. The peak is reached in the second and third weeks when the febrile reaction has ordinarily subsided and the acute phase is passed. Agglutination of the patient's own cells occurs and in some instances hemolytic anemias and similar effects have been noted. It has been suggested that the reaction indicates that the cold agglutinins are related to a specific and fundamental property of the causative agent of atypical pneumonia which is antigenically related to constituents of the human erythrocytes (121). The relationship to clinical disease has recently been reviewed by various authors (110, 122, 123, 124).

Another reaction of considerable interest is that described by Thomas and his co-workers (125, 126) who demonstrated that a nonhemolytic streptococcus (MG) was agglutinated by the serum of a relatively high proportion of convalescent atypical pneumonia patients. They have demonstrated that the reaction reaches its height in the fourth to fifth week of infection when convalescence is ordinarily well under way; that the titer is most marked in cases of severe and prolonged illness. It has also been shown that the occurrence of the agglutination of streptococci and of erythrocytes is most marked in the same patients. The reaction apparently takes place with the capsular polysaccharides of the organism and "Quellung" is observed. Agglutination of "R" variants is also seen. The authors have shown that agglutinin for erythrocytes can be absorbed without necessarily disturbing streptococcal agglutinins. Nor was the streptococcal agglutinin removed by absorption with normal animal tissue, a procedure which removes red cell agglutinins and a complement-fixing antibody which reacts with a crude suspension of normal tissue, especially that from mouse lung (127).

That these various reactions with such dissimilar materials occur in the serum of patients suggests a peculiarity of the serum. On the other hand, the possibility continues that antigenic similarity between a causative agent and these substances exists. Nevertheless, heterologous reactions do not indicate that the same substance is present in the reacting materials but may be dependent merely on the presence of a small characteristic group (128). It may be that the observations of Eaton and his associates on neutralizing antibodies and those of Horsfall *et al.* on the capacity of the convalescent sera to prevent antibody production in animals against the PVM, involve the same mechanisms as those participating in the agglutination of red cells and streptococci. Thus,

while data continue to indicate the probability that a virus is the primary causative agent of the disease with which primary atypical pneumonia is most commonly associated, both that evidence and the meaning of the serological reactions need further clarification.

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VIRUS LABORATORY, DEPARTMENT OF EPIDEMIOLOGY
SCHOOL OF PUBLIC HEALTH
UNIVERSITY OF MICHIGAN
ANN ARBOR, MICHIGAN

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